Avian Diseases

MAY 1958

Histological reaction following transplantation of chicken lymphoma (RPL-16) ..... JACK E. MOULTON AND LOGAN M. JULIAN 123

Lyophilization of avian pleuropneumonia-like organisms ..... R. D. CONRAD 132

Influence of temperature of incubation of embryonating eggs following inoculation of Newcastle disease virus ..... S. K. SINHA 138

A direct enumeration of pleuropneumonia-like organisms. C. H. DOMERMUTH 147

Experimental fungous infections in chickens. H. L. CHUTE AND D. C. O'MEARA 154

Further observations concerning Rous virus neutralizing antibodies and neoplasia. I. In farm flocks. S. G. KENZY, R. D. CONRAD AND D. M. FLUHARTY 167

Transmission and control of infectious sinusitis in turkeys. DALE J. RICHEY, B. D. BARNETT, M. A. BOONE AND C. L. MORGAN 173

The effect of antibiotics on the infectious sinusitis agent of turkeys: Part I. Egg transmission ..... O. H. OSBORN AND B. S. POMEROY 180

The acute toxicity of procaine penicillin G and of dihydrostreptomycin sulfate in the pigeon and the chicken ..... J. E. GRAY AND A. PURMALIS 187

A tissue culture-modified Newcastle disease virus I. Modification, propagation and characteristics of the tissue culture Newcastle disease virus. ..... R. A. BANKOWSKI 197

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HISTOLOGICAL REACTION FOLLOWING TRANSPLANTATION OF CHICKEN LYMPHOMA (RPL-16)*

By JACK E. MOULTON and LOGAN M. JULIAN

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Received 6 Dec 1957

THE responses of chickens to cellular implants of transplantable lymphoma (RPL 16 and similar strains) are of three types: (1) progressive growth of a local tumor followed by metastasis and death of the host, (2) local growth with subsequent regression and, (3) absorption of inoculum without tumor formation. Regression of chicken lymphoma is associated with production of humoral antibodies and with phagocytosis and necrosis of tumor cells.[2,6]

This is a report of a study of the reaction of young White Leghorn chickens to cellular transplants of RPL 16. Necrosis and phagocytosis of tumor cells in primary tumors and phagocytosis of tumor cells in metastatic sites were observed, thus confirming the observations of other workers.[6] In addition, an affinity for fluorescent dye by phagocytized tumor cells was encountered. It was possible to correlate histological reaction and fluorescence of tumor cells, with duration of the tumor within the host. Preliminary attempts to correlate cytotoxic activity of the sera of some birds with the response of the tumor are reported.

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MATERIALS AND METHODS

Tumor and Experimental Animals.—Transplantable chicken lymphoma strain RPL-16, obtained from the Regional Poultry Laboratory at East Lansing, Michigan, was employed. Characteristics of this tumor have been described.[3,5] Fifty-four birds of the 190th to 199th serial passages of RPL-16 in California were used. These were selected to include birds showing protracted survival periods. The average age was 12 days at the time of inoculation. Tumors were implanted into pectoral or right tibial muscles according to methods previously described.[5] Forty-four birds received one inoculation of tumor and 10 were injected two and three times at 3 to 6 week intervals. The time between tumor implantation and testing of tissues varied from 4 to 22 days. Birds overwhelmed by tumors in a short period of time were killed immediately before death. Others, showing protracted survival in comparison to the majority of birds of the particular passage were killed at varying intervals up to 22 days after tumor implantation. All birds were killed by decapitation, exsanguinated and necropsied immediately.

Histopathological Technique.—Tibial and pectoral tumors, spleen, liver and kidneys were fixed in 10 percent formalin, sectioned and stained with hematoxylin-eosin, Giemsa and methyl green-pyronine stains. Unfixed tissues were sectioned with a freezing microtome, mounted and fixed by incubation at 37 C for one hour. These were stained with fluorochrome dye consisting of fluorescein isocyanate coupled according to the method of Coons and Kaplan[4] to serum proteins of rabbits hyperimmunized against normal chicken globulin. The globulin fraction of normal chicken serum was obtained by precipitation at 1.39 M ammonium sulfate concentration. The globulin precipitates were redissolved in isotonic saline and the contaminating ammonium sulfate removed by dialysis against distilled water. Paper electrophoretic analysis following precipitation revealed that only traces of globulin remained among unprecipitated proteins. The rabbit serum against chicken gamma globulin was tested

by precipitation reaction with normal chicken gamma globulin and positive reactions were observed through 10^{-3} dilutions of gamma globulin after 20 minutes and 10^{-6} after 12 hours.

Sections were also stained with similar conjugates prepared with rabbit sera against *Anaplasma marginale* and *Brucella suis* and with a saline solution of 2 percent fluorescein disodium salt.

The fluorochrome procedure consisted of covering tissues with a few drops of fluorescein solution and incubating at room temperature for 30 minutes. Sections were rinsed in saline 10 minutes, mounted in glycerol-saline (9:1) and examined for fluorescence with an ultra-violet microscope.[7] Normal chicken tissues were used as controls.

Tissue Cultures.—Tumor cells obtained from primary tibial tumors of birds dying acutely of leukosis were grown in tissue culture in order to test *in vitro* action of fluorochrome dyes. Only primary explantations were used and these were added to medium consisting of 2 percent chick embryo extract, 30 percent chicken serum and 68 percent Medium 199 (Difco). Satisfactory growth was also obtained using 2 percent chick embryo extract, 15 percent horse serum ultrafiltrate and 83 percent Medium 199. Penicillin, 100 units per ml and streptomycin, 50 gamma per ml were added to control contaminants. Tumor cells were grown on glass cover slips in 5 ml shell vials. After several days of growth at 37C, cover slip cultures were removed and fixed in acetone for 10 minutes. Tissue cultures were stained with fluorochrome dyes and Giemsa stain in the same manner as tissue sections.

Normal chicken gamma globulin, normal chicken whole serum and serum from leukosis-recovered birds were added in final concentration of 20 percent to established cultures in order to test specificity of anti-chicken gamma globulin fluorescein conjugate. Cover slip cultures were removed after incubation at 37C for 24, 72 and 96 hours and stained with fluorescent conjugate.

Test for Cytotoxic Activity.—Sera of five birds that survived for protracted periods were tested for humoral cyto-

toxins against tumor cells by a modification of the method of Burmester.[1] Blood containing tumor cells from donor birds bearing tumor implants was washed twice and reconstituted to original volume in Ringer's solution. Ten percent (V/V) tumor cell suspensions were prepared containing final serum concentrations of 4 and 20 percent. Dilutions were made with Ringer's solution. Controls were prepared using serum of a bird that had not been exposed to leukosis and whose serum repeatedly failed to demonstrate cytotoxic effect against tumor cells. Test and control samples were incubated at 37C for 22 hours and 0.25 ml aliquots were inoculated intraperitoneally into susceptible chicks less than one week of age. Ten chickens were employed for each sample tested and the presence of cytotoxin was determined by comparing the percentage of deaths of chicks with typical lesions of leukosis in test and control groups and by comparing average survival periods of chicks in the two groups. In the case of a serum sample containing cytotoxin, one would expect fewer test than control chicks to die and longer survival than controls of those that did die.

RESULTS

Histological Reaction.—Fourteen birds that survived tumors 9 days or longer and four birds in the 6 to 8 day group exhibited histological reaction to tumors (figure 1). Sera of five of these birds were tested for cytotoxic activity against tumor cells and significant titers were demonstrated in two. There was no correlation between presence of cytotoxin at detectable levels and age of the bird, size of tumor, age of implant or previous experience with tumor.

The histological reaction consisted of focal necrosis of primary tumor and phagocytosis of tumor cells by macrophages in primary and metastatic sites. Necrosis was observed in primary tumors of all but one bird showing protracted survival time, but was not present in metastatic sites in liver, spleen and kidney. Phagocytic cells were scattered throughout primary tumors, unrelated to necrotic areas, and were especially numerous in metastatic sites. Phagocytized tumor cells numbered two to four within a macrophage and

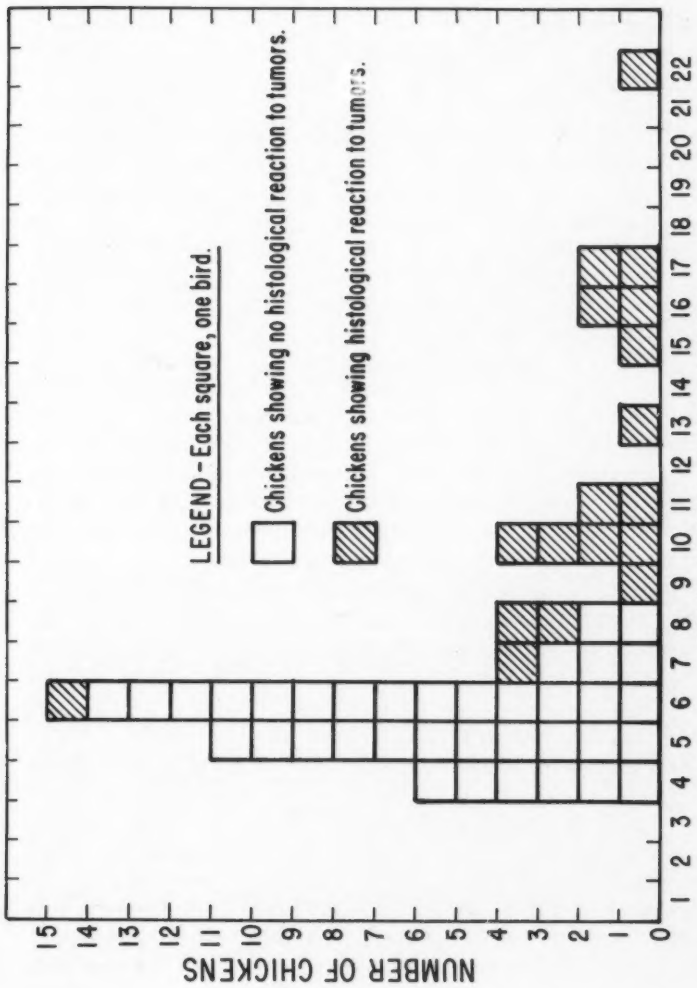

Fig. 1. Reaction of chickens after inoculation of RPL16 chicken lymphoma.

often exhibited hyperchromatic nuclei (figure 2). Tumor cells revealed no abnormal changes prior to phagocytosis. Phagocytosis was prominent in splenic sinuses, hepatic sinusoids and between renal tubules (figure 3). Tumor cells were phagocytized principally by Kupffer cells in the liver. Right kidneys of 13 birds showed metastatic tumor cells, many of which were phagocytized, while left kidneys of only three birds were involved. Spleens of seven birds that showed phagocytosis exhibited hyperplasia of reticuloendothelial cells around sheathed arteries and spleens of four birds displayed atrophy of lymph follicles. Plasma cell proliferation could not be correlated with phagocytosis in tissues stained with methyl green-pyronine.

Birds overwhelmed with lymphoma in 8 days or less revealed extensive tumor invasion in primary and metastatic sites and right kidneys were involved more often than left. None of these showed necrosis or phagocytosis of tumor cells.

Fluorescence of Tumor Cells.—An unusual finding observed only in birds resisting tumors was the bright, yellowish-green fluorescence of tumor cell nuclei in primary and metastatic sites. Free and phagocytized tumor cells showed fluorescence in primary tumors while only phagocytized cells fluoresced in metastatic areas (figures 4, 5). Phagocytosis could be detected more accurately in fluorochrome sections than in hematoxylin-eosin and Giemsa stained sections. The same degree of fluorescence was observed in sections stained with fluorescent anti-chicken globulin serum, fluorescent *Anaplasma marginale* and *Brucella suis* sera, and fluorescein dye in saline solution. Normal birds and birds that died in 8 days or less displayed no cellular fluorescence.

Tumor cells grown in tissue culture and removed at varying intervals even after degeneration of tumor cells occurred, exhibited no specific fluorescence when stained with fluorochrome dye. Tissue cultures incubated at varying intervals in chicken gamma globulin, normal chicken whole serum and serum from leukosis-recovered birds and subsequently stained with anti-chicken gamma globulin fluorescein conjugate, revealed no specific fluorescence of tumor cells.

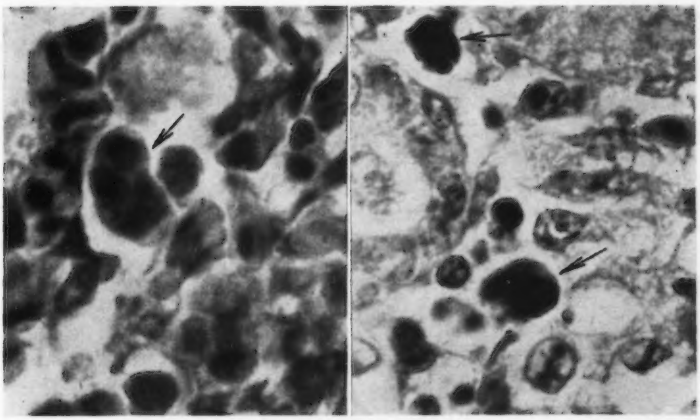

Fig. 2 (left). Splenic red pulp. Arrow points to a group of tumor cells within a macrophage. Cells such as these exhibit fluorescence when stained with fluorochrome dyes. Hematoxylin and eosin stain. × 960.

Fig. 3 (right). Macrophages with phagocytized tumor cells (see arrows) between tubules of the kidney. Hematoxylin and eosin stain. × 960.

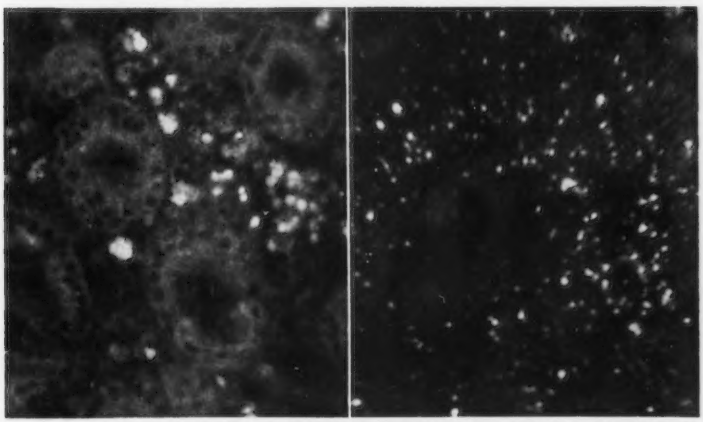

Fig. 4 (left). Fluorescent tumor cells within macrophages between tubules of the kidney. Fluorochrome dye. × 360.

Fig. 5 (right). Spleen showing fluorescent tumor cells within red pulp. The fluorescent tumor cells are phagocytized by macrophages. Fluorochrome dye. × 180.

DISCUSSION

It appears that apparently intact tumor cells in chickens surviving a long time become phagocytized by macrophages and exhibit specific fluorescence when stained with fluorescein dye. Birds dying in shorter periods of time show neither phagocytosis nor fluorescence of tumor cells. This confirms the work of Love and Sharpless[6] with regard to phagocytosis of tumor cells in avian lymphomatosis and correlation of this with duration of tumor growth. They interpreted phagocytosis as part of a reaction resulting in regression of lymphoma and our findings agree. It is likely that this reaction is associated with an immunological process in the host. In fact, it has been shown that chickens which survive initial implants of this tumor show resistance to subsequent implants, and that antibody may be demonstrated in sera of these immune birds by its *in vitro* toxic effects upon tumor cells and by its *in vivo* effect when injected into birds before and after receiving tumor cells.[1,2] Titrations of sera from two resistant birds in the present study revealed definite antitumor cell activity. Love and Sharpless[6] observed hyperplasia of plasma cells in spleens of resistant birds that showed phagocytosis of tumor cells and interpreted this in the light of antibody production.

Fluorescence of tumor cells observed in the present study was not due to gamma globulin coating tumor cells for no difference in staining was noted in sections treated with anti-chicken gamma globulin conjugate and with non-specific conjugates and fluorescein in saline solution. In addition, tumor cells in tissue cultures incubated with chicken gamma globulin and subsequently stained with anti-chicken gamma globulin fluorescein conjugate exhibited no specificity of staining. However, it seems likely that phagocytized tumor cells are altered in some manner either before or after phagocytosis so that they stain specifically with fluorochrome dye. This may involve a fluorescein stainable substance coating the cells or increased permeability of cells to dye. Fluorescence of free cells in primary tumors could be due to degenerative changes altering permeability of some of the older cells, but no such

changes were detected in hematoxylin-eosin and Giemsa stained sections. Necrotic tumor cells in sections of primary tumors stained with usual stains did not occupy the same location as fluorescent cells in immediately adjacent sections. Also, cells grown in tissue culture and stained after varying incubation periods including stages of degeneration revealed no specific fluorescence.

The greater degree of phagocytosis and fluorescence of tumor cells in right kidneys as compared to left is due to the renal portal system of the chickens which allows more tumor cells to metastasize to the right kidney from right tibial implants.[5] These cells being in the area longer, have greater opportunity to become phagocytized.

SUMMARY

Regression of transplantable lymphoma RPL-16 was accompanied by phagocytosis and fluorescence of tumor cells. Tumor cells from birds with rapidly growing tumors exhibited neither phagocytosis nor fluorescence.

REFERENCES

1. Burmester, B. R. The cytotoxic effect of avian lymphoid tumor anti-serum. Cancer Res., *7*, 459, 1947.
2. Burmester, B. R., and C. O. Prickett. Immunity reaction obtained with a transmissible fowl tumor (Olson). Cancer Res., *4*, 364, 1944.
3. Burmester, B. R., and C. O. Prickett. Development of highly malignant tumor strains from naturally occurring avian lymphomatosis. Cancer Res., *5*, 652, 1945.
4. Coons A. H., and M. H. Kaplan. Localization of antigen in tissue cells. II. Improvement in a method for the detection of antigen by means of fluorescent antibody. Jour. Exp. Med., *91*, 1, 1950.
5. Julian, L. M. Effect of transplantable lymphomatosis (Strain RPL 16) on young rapidly growing chickens. Am. Jour. Vet. Res., *14*, 601, 1953.
6. Love, R., and G. R. Sharpless. Studies on a transplantable chicken lymphoma (RPL-12). I. Pathologic changes following transplantation of the tumor. Cancer Res., *13*, 869, 1953.
7. Moulton, J. E., and J. A. Howarth. The demonstration of *Leptospira canicola* in hamster kidneys by means of fluorescent antibody. Cornell Vet., *47*, 524, 1957.

LYOPHILIZATION OF AVIAN PLEURO-PNEUMONIA-LIKE ORGANISMS*

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Received 12 Dec 1957

GREY and Turner[4] have studied the viability and immunizing potency of freeze-dried, bovine pleuropneumonia, culture vaccines. They observed that approximately one percent of the population survived the lyophilization process after 41 months storage at 4 C.

This study is concerned with the effects of the lyophilization process on the viability of avian pleuropneumonia-like organisms (PPLO).

MATERIALS AND METHODS

Four avian PPLO isolates were used in these experiments. The isolate designated RIP, of the State College of Washington collection, had been obtained from a field case of chronic respiratory disease (CRD) by H. E. Adler in 1952. This isolate had been adapted to artificial culture medium at the time when these experiments were instigated. Three isolates, lethal for chick embryos obtained from J. Fabricant, were designated CKAU, CB and CU. At the time of this investigation, isolate CKAU represented the 125th egg-passage,

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**The writer expresses appreciation for the technical assistance of Mr. Roy Wright and Mrs. W. Rickard.

isolate CB the 201st egg passage, and isolate CU the 44th egg-passage.

The media used for the propagation of RIP isolate were Difco PPLO broth without crystal violet and Difco PPLO agar. Both were supplemented to 2 percent by volume with Difco PPLO serum fraction. Penicillin was added to broth and agar to a final concentration of 1,500 units per ml.

Fertile chicken eggs were obtained from the Poultry Department of the State College of Washington. No respiratory disase had been observed in this flock. Serum samples from the laying hens were devoid of PPLO antibodies, as ascertained by the serum-plate agglutination test.[1]

Isolates CKAU, CB and CU were propagated in the yolk sacs of 5-day, embryonating chicken eggs. The inoculum consisted of 0.05 ml of undiluted material. After 48 hours incubation, all of the inoculated chicken embryos were killed. Yolk materials were harvested on the day of embryonic death. Samples of yolk were streaked on blood agar and inoculated in thioglycolate medium to detect bacterial contamination. Contaminated yolk samples were discarded. Uncontaminated yolk samples were pooled and held at –20 C. PPLO could be cultivated on supplemented PPLO agar from the yolk material in which these isolates had been prepared.

PPLO population determination as detected by plate counts.—The total viable population of RIP isolate was determined by means of a direct plating technique. Fivefold serial dilutions were made of broth culture, using sterile 0.5 percent sodium chloride solution buffered at pH 7.2. One-tenth ml of each dilution was placed in a sterile 5 cm petri plate. Three ml of supplemented PPLO agar, cooled to 45 C, was added to each petri plate and the contents mixed by gentle agitation. These plates were prepared in duplicate and incubated for 96 hours at 37 C. Only plates containing more than 30 or less than 300 PPLO colonies were counted. A culture plate was wedged in a small 5.2 cm square box with 0.5 cm wire grid and examined under a stereoscopic wide field microscope. To facilitate counting, the colonies were

stained *in situ* using Unna's polychrome methylene blue (Harleco) diluted 1:200 with distilled water. Both surface and subsurface PPLO colonies were stained the characteristic deep blue while contaminants were stained a light magenta.

One-tenth ml aliquots of the broth culture of this isolate were transferred aseptically to sterile pyrex 8 mm x 130 mm lyophilizing tubes. These samples were shell-frozen in a dry ice and absolute alcohol mixture, attached to a freeze-dry apparatus and allowed to remain for 6 hours after initial apparent drying had been accomplished. The pressure varied from 10 to 50 microns of mercury after the initial sublimation had been completed. The tubes were removed from the machine by sealing under vacuum with an oxygen gas torch and were tested for vacuum using the high-voltage spark apparatus.

Immediately after lyophilization, two tubes were opened and the contents removed with four washings of sterile PPLO broth to a volume of 2 ml. Fivefold serial dilutions of these lyophilized aliquot samples were prepared in duplicate and viable counts were determined as previously described. Lyophilized samples were stored at 4 C.

LD_{50} titer reduction after lyophilization.—Five-hundredths ml of yolk suspension from each of the three isolates, CKAU, CB and CU, was inoculated in 5-day embryonating chicken eggs via the yolk sac. Samples were serially diluted tenfold through the 10^{8} dilution. Each dilution was inoculated in a group of ten eggs. Four ml aliquot samples of the serial dilutions were aseptically removed and placed in sterile 25 ml Stokes lyophilizing tubes, shell-frozen in an absolute alcohol and dry ice mixture and then attached for lyophilization. Tubes were removed from the machine under vacuum with an oxygen gas torch 6 hours after initial sublimation was evident. Ninety-six hours after lyophilization, these tubes containing the diluted yolk material were opened and each reconstituted with 4 ml of sterile distilled water. Five-hundredths ml of the aliquot samples of each dilution was inoculated into 10, 5-day chicken embryos via the yolk

sac. LD_{50} titers were determined by the Reed-Muench[7] method. The eggs were candled daily through the 18th day of incubation and deaths recorded. Controls consisted of chicken embryos inoculated with 0.05 ml of pH 7.2, sterile, phosphate buffered, 0.5 percent NaCl. Only embryo deaths after 72 hours incubation were used in the accumulative death totals.

RESULTS

PPLO population determination as detected by plate counts.—The results of this experiment are summarized in table 1. Unlyophilized samples of the culture were found to contain an average of 1,480,000 viable organisms per ml. The aliquot samples, opened immediately after lyophilization, contained an average number of viable cells representing 23.8 percent of the original population. The totals shown in table 1 represent the average of duplicate counts. The population of RIP isolate was enumerated and aliquot samples lyophilized on December 29, 1953. Two lyophilized tubes were opened at 1, 20, 57, 382 and 831 days after lyophilization, and the viable populations determined for each period.

LD_{50} titer determination after lyophilization.—The results of this experiment are summarized in table 2. The medial lethal doses for 50 percent of the test subjects suggest that a difference of virulence existed among the three isolates studied. This was demonstrated by LD_{50} log titers of 7.36 (CKAU), 5.35 (CB) and 6.55 (CU). Log titer reductions of the lyophilized aliquots were found to be 1.43 (CKAU), 1.48 (CB) and 1.13 (CU).

DISCUSSION

The results of these experiments reveal that four strains of PPLO originating in chickens survived the freeze-dry process. The variation in counts (table 1) may be the combined result of two factors: first, errors in the counting techniques; second, the difference of survival rates of the population in tubes at various positions on the apparatus.

TABLE I

The survival of lyophilized PPLO (RIP isolate) as determined by direct plate counts. The original non-lyophilized culture contained 1.48×10^6 organisms per ml.

Number days after lyophilization	Tube	Duplicate counts per tube	Average counts	Average percent survival
1	A	265,000 286,000	275,000	18.6
	B	240,000 238,000	238,000	16.1
20	C	465,000 433,000	449,000	30.3
	D	365,000 342,000	353,000	23.8
57	E	325,000 312,000	318,000	21.5
	F	375,000 341,000	358,000	24.2
382	G	500,000 500,000	500,000	33.7
	H	300,000 310,000	305,000	20.6
831	I	386,000 370,000	378,000	25.5
	J	260,000 218,000	239,000	16.1

TABLE II

The effect of lyophilization on lethality for chicken embryos of three isolates of PPLO as indicated by LD_{50} titers

Strain	LD_{50} titer before lyophilization	LD_{50} titer after lyophilization	Log dilution reduction
CKAU	$10^{-7.36}$	$10^{-5.93}$	1.43
CB	$10^{-5.35}$	$10^{-3.87}$	1.48
CU	$10^{-6.55}$	$10^{-5.42}$	1.13

Furthermore, survival rates would depend upon the lyophilization techniques, which vary with each run.

Forty-four PPLO isolates of our stock collection have also been lyophilized. All tubes opened over a 14-month period have contained viable organisms.

It has been demonstrated that a part of the PPLO population of egg-propagated avian respiratory vaccines could survive a lyophilization process. The possible relationship of live virus vaccines in the spread of CRD has caused concern among certain poultry disease investigators. That virus infection may be associated with CRD has been suggested by Adler,[2] hence the significance of contaminating egg-propagated avian respiratory virus vaccines becomes evident. This hazard was revealed earlier by the demonstration of avian PPLO in eggs[3] and the establishment of egg transmission of CRD[5] and infectious sinusitis.[5,6] The survival of this group of organisms, if present in a vaccine, could result in the dissemination of CRD. However, unpublished experience in our laboratory indicates that many of the isolates observed are of doubtful pathogenicity. The presence of the respiratory virus must be considered as being potentially capable of producing conditions whereby the infectious dose of a PPLO could effectively be lowered. If present, PPLO would probably be in low numbers. The lyophilization process would lower that population. Further investigations would be needed to determine the significance of these factors.

SUMMARY

1. An isolate of pleuropneumonia-like organism of avian origin, adapted to artificial cultural medium was lyophilized. Approximately 23.8 percent of the population remained viable after 27 months storage at 4 C.

2. Of three isolates of pleuropneumonia-like organisms lethal for embryonating chicken eggs, the LD_{50} log titers were reduced 1.13, 1.48 and 1.43, 96 hours after lyophilization.

3. The possible relationship of avian respiratory vaccines contaminated with pleuropneumonia-like organisms to the spread of chronic respiratory disease is discussed.

REFERENCES

1. Adler, H. E. A rapid slide agglutination test for the diagnosis of chronic respiratory disease in the field and in laboratory-infected chickens and turkeys—a preliminary report. Proc. Am. Vet. Med. Assoc., 91st Ann. Mtg., 346, 1954.

2. Adler, H. E. A study of pleuropneumonia-like organisms associated with chronic respiratory disease of chickens and turkeys. Thesis, Univ. of California, 1955.
3. Cover, M. S. and E. F. Waller. The presence of chronic respiratory disease in pipped eggs. Am. Jour. Vet. Res., *15*, 119, 1954.
4. Gray, D. F. and A. W. Turner. Viability and immunizing potency of freeze-dried bovine contagious pleuropneumonia culture-vaccine. Jour. Comp. Path., *64*, 116, 1954.
5. Jerstad, A. C., C. M. Hamilton and V. E. Smith. Preliminary report of probable egg transmission of infectious sinusitis in turkeys. Vet. Med., *44*, 272, 1949.
6. Jerstad, A. C. Personal communication, 1955.
7. Reed, L. J. and H. Muench. A method for estimating fifty percent end points. Amer. Jour. Hyg., *27*, 493, 1938.
8. Van Roekel, H., L. P. Beninato and O. M. Olesiuk. Egg transmission of chronic respiratory disease agent (preliminary report). Proc. 26th Ann. Conf. Laboratory Workers in Pullorum Disease Control, June 14-15, 1954.

INFLUENCE OF TEMPERATURE OF INCUBATION OF EMBRYONATING EGGS FOLLOWING INOCULATION OF NEWCASTLE DISEASE VIRUS

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Received 12 Dec 1957

IT HAS been known that the age of embryonated chicken eggs and the temperature of incubation have an effect on development of lesions with variations from one virus to another. Furthermore, an optimal incubation temperature as well as an optimal age of embryo seems to exist for each virus

infection in the embryonating egg.

While attempting to purify influenza virus, McLean, *et al.*,[5] studied the rate of increase in infectivity and hemagglutination activity of the influenza virus B (Lee strain) in the chorioallantoic fluid of chick embryo incubated for varicus periods at 35, 37, and 39 C. The maximal level of infectivity was reached at 35 C following 48 hours of incubation, and was about six times that attained at 37 C after 36 to 48 hours incubation. They also found greater HA activity at 35 C than at 37 C and no evidence of activity at 39 C. Sigurdson[7] similarly demonstrated that better multiplication of influenza virus can be obtained at 35 C to 36 C than at 39 C to 40 C.

With the effect of temperature of incubation on virus infections in 12 day old embryonated eggs, Burnet and Lush[2] and Burnet[1] observed in ectromelia virus that pocks developed much better at 36 C to 37 C than 38.5 C or 39.5 C; with vaccinia and variola only minor lesions developed on the membranes at 28 C in contrast to 37 C where marked lesions are found.

The effect of age of embryos on the pathogenesis of virus infections in the egg have been observed by Shaffer and Enders[6] where they found that the embryos younger than 10 days do not develop marked lesions on the membrane when inoculated with herpes virus.

The host parasite relationship has been previously studied (Sinha, *et al.*,[8]) employing only the virulent strain GB of Newcastle disease virus (NDV) in the chickens. In this paper, an attempt has been made to study the effect of temperature and age on a virus infection of an immature host, the embryonating egg.

MATERIALS AND METHODS

Virus: In this present study, strains of high, moderate and low pathogenicity were chosen such as GB, Herts, KM, Roakin, NaJ and B_1. According to Hanson,[4] the strains GB and Herts are velogenic, the strains KM, Roakin and NaJ are mesogenic types and the B_1 is a lentogenic strain. A complete history of the strains was described by Hanson.[4]

Source of eggs: The fertile eggs were from a flock that had not been affected with Newcastle disease. Upon arrival the eggs were incubated at 99 F. Later, the eggs were inoculated at the desired age with the selected strain of NDV and acclimated at desired incubation temperatures. A Jamesway egg incubator was used in which the temperature varied 0.5 C.

In the first part of this experiment, the strains GB, Herts, KM, Roakin, NaJ, and B_1 of NDV were inoculated in the allantoic chamber of 10-day-old embryonating eggs. Ten embryos were inoculated per dilution and each embryo received 0.2 ml of the inoculum into the allantoic chamber.

In the second part, the Roakin strain was inoculated intra-allantoically in 7, 10, and 12-day-old embryonating eggs. The same amount of inoculum and the same number of eggs were used per dilution as in part I.

The eggs were candled at intervals of 4 to 6 hours following inoculation. Deaths of embryos were considered to be due to virus infection when the harvested allantoic fluid possessed HA activity.

RESULTS

The mean number of hours for death of 10 day embryonating eggs following incubation of GB, Herts, KM, Roakin, NaJ and B_1 strains of NDV incubated at 99 F and 95 F are shown in table I. The difference in time of embryonic death at 99 F with that at 95 F was significant. The embryonic death was earlier at 99 F than at 95 F in all corresponding log dilutions of the virus. Considering all log dilutions tested at 99 F and 95 F incubation temperatures, the differences in mean death time between two temperatures are as follows: GB—9 to 15 hours; Herts—9.2 to 12.2 hours; KM—11.6 to 12.6 hours; Roakin—8.5 to 14.8 hours; NaJ—10.4 to 11 hours; and B_1—10 to 11 hours. The above differences are shown in figure 1.

The results of the influence of incubation temperatures on the death of 7, 10 and 12 day old embryonating eggs following inoculation with the Roakin strain of NDV are given in table 2. The mean hour of embryonic death was much earlier in 7 day old embryos than in 10 and 12 day embryos

TABLE I

Effect of incubation temperature on death of embryonating eggs inoculated with GB, Herts, KM, Roakin, NaJ and B_1 strains of Newcastle disease virus.

	Mean number of hours for death of 10-day-old embryonating eggs											
LD_{50} of virus	GB		Herts		KM		Roakin		NaJ		B_1	
	99 F	95 F	99 F	95 F	99 F	95 F	99 F	95 F	99 F	95 F	99 F	95 F
10^1					46.4	58.0	54.0	62.5	46.8	57.2		
10^2	40.0	49.0	38.4	47.6							108.0	119.0
10^3					43.2	52.3	46.6	60.4	43.2	52.4		
10^4	37.0	47.3	36.8	46.6							92.2	110.0
10^5					36.0	47.2	41.6	52.8	38.4	47.2		
10^6	33.0	45.1	32.0	45.0							89.8	94.0
10^7					31.4	44.0	36.0	50.4	34.6	45.6		
10^8	28.5	43.6	27.2	42.4							80.0	90.8

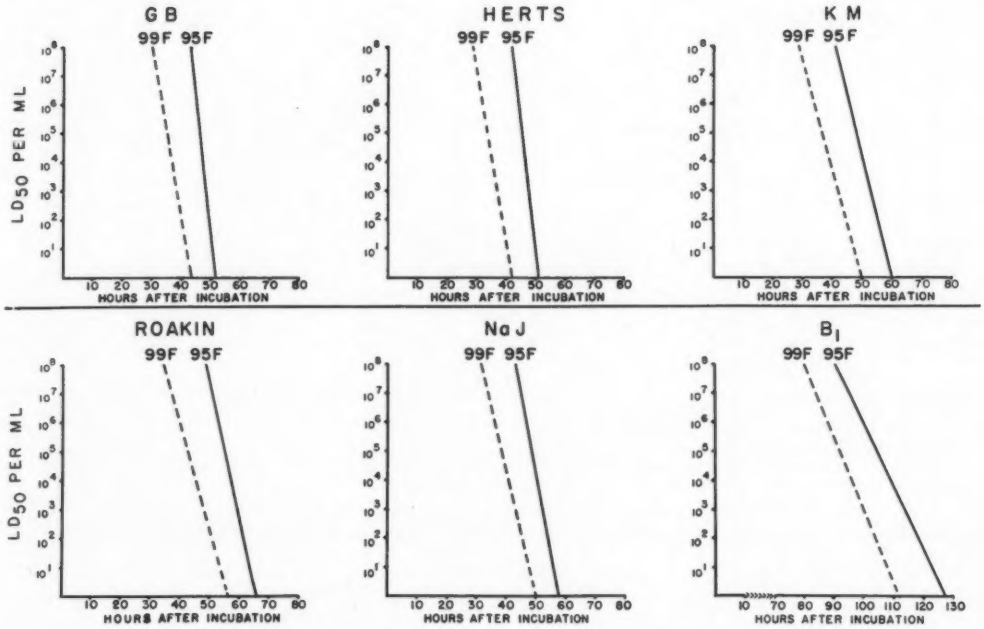

Fig. 1. The effect of incubation at 99 F and 95 F on death of 10 day embryos inoculated with GB. Herts, KM, Roakin, NaJ and B_1 strains of Newcastle disease virus.

at both incubation temperatures (figure 2). The difference in hour of embryonic death was also observed between the two incubation temperatures at different log dilutions of Roakin strain. The embryos died much earlier at 99 F than at 95 F in all three ages of embryos tested (figure 3).

TABLE II

Effect of incubation temperature on death of 7, 10, and 12 day old embryonating eggs inoculated with the Roakin strain of NDV.

LD_{50} of virus	Mean number of hours for death of embryonating eggs					
	7 day embryos		10 day embryos		12 day embryos	
	99 F	95 F	99 F	95 F	99 F	95 F
10^{1}	46.0	56.4	54.4	62.8	57.4	71.0
10^{3}	37.2	50.0	46.2	60.5	48.1	62.8
10^{5}	35.2	42.4	41.3	52.9	44.0	54.8
10^{7}	32.8	37.3	36.2	50.1	41.2	51.0

DISCUSSION

The above results are interesting in view of previous reports made by other workers on the effect of incubation temperature on the pathogenesis of virus infection in the embryonating eggs. Burnet[1] observed that egg adapted influenza virus in 12 day embryos seemed to be more virulent at 36 C to 37 C than at 38.5 C. Enders and Pearson[3] showed that influenza virus could be demonstrated in 15 day embryos after 3 days' incubation at 37 C but not after 5 days. When the eggs were incubated at 41 C it could not be found after 2 days. With the virus of ectromelia, 12 day embryos usually died in 4 to 5 days at 36 C to 37 C. However, the embryos survived when incubated at 39.5 C.[2]

It seems that an optimal incubation temperature as well as an optimum age of a host exist for each virus infection. The host parasite relationship varies from one virus to the other. In case of NDV, the slower rate of death of the embryos has been observed at the lower incubation temperature (95 F) than at higher (99 F) incubation temperature. In this situation, there are factors worth considering.

The degree of differentiation and maturation of 7 day embryos from that of 12 day old is quite well known. As the

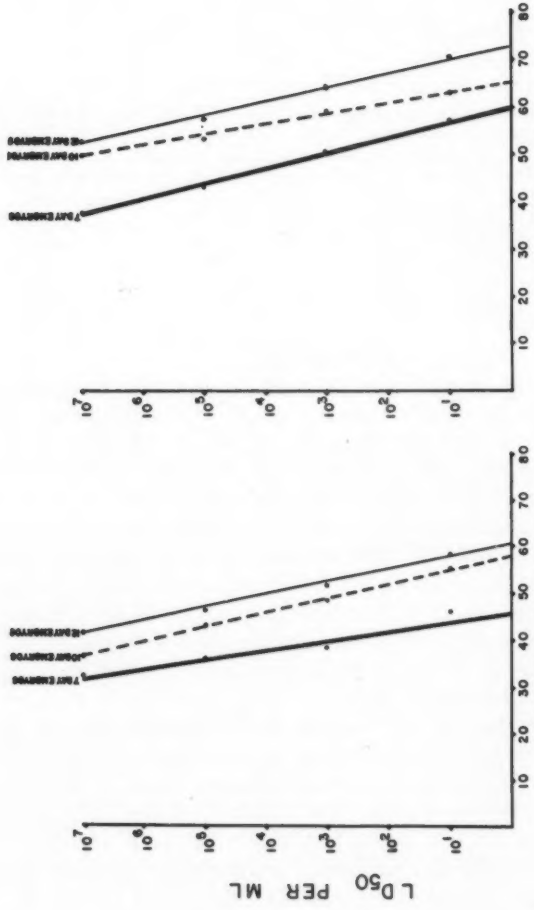

Fig. 2. The effect of incubation at 99 F and 95 F on time of death of 7, 10, and 12-day-old embryos inoculated with Roakin Newcastle disease virus.

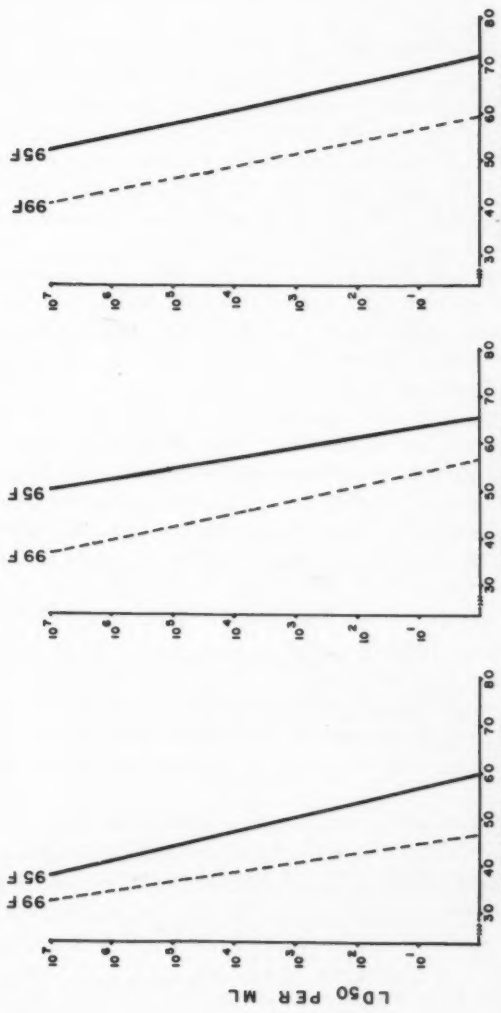

Fig. 3. The effects of incubation at 99F and 95F following allantoic inoculation of Roakin strain NDV on 7, 10, 12-day-old embryonating eggs.

host grows older, the leucocytes, macrophages appear, blood proteins develop and fundamental metabolic changes take place. On the other hand, the multiplication of the virus in the embryonating eggs at the lower incubation temperature may be delayed due to slower growth rate of the embryo. The slower growth would result from the low metabolic activity of the cells at a low temperature while at the higher temperature (99 F), the metabolic rate in the embryonating egg would favor rapid growth and multiplication of the virus and thereby result in earlier death of the embryo.

Furthermore, it is logical to assume that certain enzymes of the cells of embryo play a particularly critical role in the growth and multiplication of the NDV. The incubation temperature may affect the utilization of such an enzyme for viral growth. In other words, lower incubation might induce " a biochemical barrier" against the parasite in the host. However, it is not known whether lower incubation temperature exerts its effect by influencing primarily the virus or the embryo. The true mechanism of resistance is not known. However, the resistance to viral infections and neutralizing antibodies in the circulation may develop independently in the immature host.

SUMMARY

A significant difference of several hours in the death of 10-day-old embryonating eggs incubated at 99 F and 95 F has been observed following inoculation of GB, Herts, KM, Roakin, NaJ, and B_1 strains of Newcastle disease virus by the allantoic route. The embryos died much earlier at 99 F than at 95 F.

Similarly, an earlier embryonic death was observed at 99 F than at 95 F in 7, 10, and 12 day old embryonating eggs following allantoic inoculation of Roakin strain of Newcastle disease virus.

REFERENCES

1. Burnet, F. M. Influenza virus on the developing egg. I. Changes associated with the development of an egg passage strain of virus. Brit. Jour. Exp. Path. *17*, 282, 1936.
2. Burnet, F. M. and D. Lush. The propagation of the virus of infectious ectromelia of mice in the developing egg. Jour. Path. and Bact. *43*, 105, 1936.

3. Enders, J. F. and H. E. Pearson. Resistance of chicks to infection with influenza virus A. Proc. Soc. Exp. Biol. and Med. *48*, 143, 1941.
4. Hanson, R. P. Identification of strains of Newcastle disease virus (in mimeograph). Department of Veterinary Science, University of Wisconsin, Madison, Wisconsin, 1955.
5. McLean, I. W., Jr., D. Bear, A. R. Taylor, D. G. Sharp, J. W. Beard, A. E. Feller, and J. H. Dingle. Influence of temperature of incubation on the increase of influenza virus B (Lee strain) in the chorio-allantoic fluid of chick embryos. Jour. Immunol. *48*, 305, 1944.
6. Shaffer, M. F. and J. F. Enders. Quantitative studies on the infectivity of the virus of herpes simplex for the chorio-allantoic membrane of the chick embryo, together with observations on the inactivation of the virus by its specific antiserum. Jour. Immunol. *37*, 383, 1939.
7. Sigurdsson, B. The influence of age of host and temperature of incubation on infection of the chick embryo with vesicular stomatitis virus. Jour. Exp. Med. *78*, 17, 1943.
8. Sinha, S. K., R. P. Hanson, and C. A. Brandly. Effect of environmental temperature upon facility of aerosol transmission of infection and severity of Newcastle disease among chickens. J. Inf. Dis., *100*, 162, 1957.

A DIRECT ENUMERATION OF PLEUROPNEUMONIA-LIKE ORGANISMS*

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MANY different techniques have been utilized in efforts to estimate numbers of pleuropneumonia-like organisms (PPLO). Prior to 1932, visual methods of estimating turbidity and colony size were frequently employed. In 1932 Holmes and Pirie using a methylene blue indicator measured the

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reduction of lactate by the agent of bovine pleuropneumonia. Smith and Morton[7,8] utilized mean colony diameters as an index of growth of pleuropneumonia-like organisms. Keller and Morton[2] utilized turbidity, the presence of growth following tube dilutions and colony counting to estimate the growth of PPLO. Liebermeister[4] counted the number of colonies which developed from a standard amount of inoculum. Lecce and Morton,[3] and Rodwell and Rodwell[5] measured and correlated PPLO cellular nitrogen to optical density in an effort to standardize material for metabolic studies and, Smith[6] reported the "measurement of turbidities of concentrated cultures, of colony diameters, and of number of viable cells by colony counting" to be useful methods for the quantitation of growth of PPLO. He reported that "measurement of acid production, of turbidities of unconcentrated cultures, and of zone growth by the cup plate method are of no value for assessment of growth".

It is obvious that a variety of methods have been utilized in an effort to estimate PPLO numbers. However, a review of the literature did not disclose any attempt to estimate PPLO numbers by direct microscopic counts of PPLO organisms. The purpose of this paper is to report the results of such an attempt.

MATERIALS AND METHODS

Passages 248 (experiment 1) and 252 (experiment 2) of the Van Roekel avian PPLO strain were used as the test organism in this experiment. The Van Roekel PPLO strain has been routinely carried on stock Difco PPLO broth without crystal violet (medium no. 1[a]). It has been transferred weekly in a 10 percent inoculum and incubated at 37 C.

Experiment 1.

A seed inoculum was prepared using medium no. 1 which had been incubated for 3 days following inoculation. Flasks

a. Bacto PPLO Broth W/O CV, 1 percent Bacto PPLO serum fraction, 1:2000 parts thallium acetate, 1000 units penicillin per ml, and distilled water.

of medium no. 1, no. 2[b], no. 3[c], and no. 4[d], (250 ml per 1000 ml flask) were inoculated with 10 percent of the seed inoculum and incubated at 37 C for 9 days. After two days of incubation 60 ml samples were removed from each of the four cultures. Viable counts in homologous media were made from 10 ml of each sample, and the remaining 50 ml amounts were concentrated 10 times by centrifugation at 14,000 times gravity for 30 minutes at 6 C and resuspended in 5 ml of sterile distilled water. One hundredth ml of this suspension was spread evenly over a one square cm circular area which had been scribed on a glass slide with a diamond pencil. This preparation was air dried, fixed in methyl alcohol for 3 minutes and stained with freshly filtered (Whatman no. 1) Giemsa stain for 60 minutes. Five oil immersion fields were counted, the center field, and fields at 12, 3, 6, and 9 o'clock positions on the slide. The latter four fields were one low power field removed from the circumference of the preparation. All four preparations were counted twice by two different persons. Optical density determinations using a Fisher electrophotometer with a 4250A filter were made on the remaining portions of the concentrated samples. After a total of 9 days of incubation time had elapsed, the entire procedure was repeated.

Experiment 2.

The procedure outlined in experiment 1 was repeated *in toto* with the exception that a faulty thermostat caused the incubator to vary $\pm$ 7 C from 37 C.

RESULTS

The PPLO cells in samples prepared for counting were uniformly distributed throughout the inscribed areas on the slides and the individual cells were stained sufficiently well to permit them to be counted. The preparations were comparatively free of extraneous matter (figure 1).

b. The basal medium (footnote a) plus 0.25 percent dextrose and phenol red.

c. The basal medium (footnote a) plus 0.25 percent Bacto dextrose, phenol red, and 1 percent yeast hydrolysate (Nutritional Biochemicals Corporation).

d. The basal medium (footnote a) plus 1 percent yeast hydrolysate.

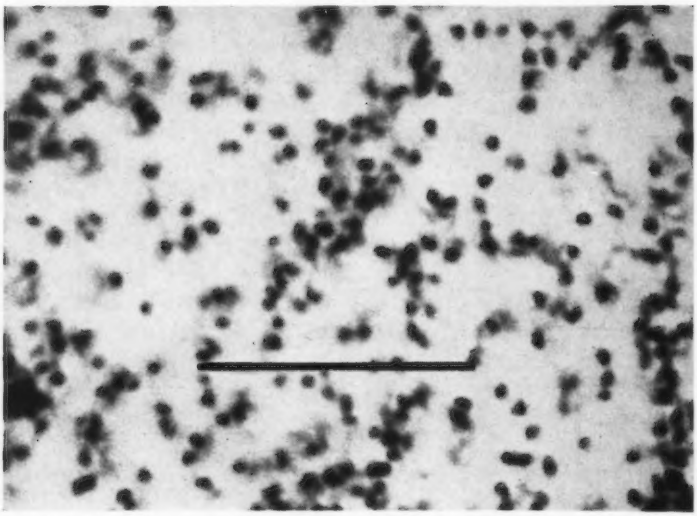

Fig. 1. Giemsa stained avian pleuropneumonia-like organisms prepared for counting. The line in the figure represents a distance of 10 microns. The majority of the cells are isolated and rather uniform in appearance. *Photography by Dr. W. B. Gross*

The direct microscopic counts obtained by the two operators were averaged for each sample and the percent differences from these means were calculated. The greatest difference from the mean was 4.4 percent, the least was 0.9 percent and the average was 2.0 percent (table I).

TABLE I

Differences in percent from the average pleuropenumonia-like organism counts for two operators counting identical samples.

Days incubation	Media*	Percent difference from mean			
		Experiment no. 1		Experiment no. 2	
		Operator 1	Operator 2	Operator 1	Operator 2
2	1	—1.0	+1.0	—2.5	+2.5
	2	—1.4	+1.4	+0.9	—0.9
	3	+1.3	—1.3	+0.6	—0.6
	4	+3.0	—3.0	+1.5	—1.5
9	1	—4.2	+4.2	+1.1	—1.1
	2	—1.7	+1.7	—3.3	+3.3
	3	—4.4	+4.4	—2.3	+2.3
	4	+1.8	—1.8	+0.9	—0.9

*See footnotes a, b, c and d (Page 148, 149) for composition of media.

The comparative estimates of numbers of pleuropneumonia-like organisms propagated in the four different types of media are shown in table II (experiment 1) and table III (experiment 2).

TABLE II

Comparative estimates of numbers of pleuropneumonia-like organisms propagated in four different media.

Days incubation	Media*	Percent** transmission	Viable Count/ML	Microscopic Count/ML
2	1	73.3	$1.0X10^{7}$	$1.1X10^{8}$
	2	46.7	$3.2X10^{5}$	$1.9X10^{8}$
	3	29.2	$3.2X10^{4}$	$1.1X10^{8}$
	4	34.3	$3.2X10^{5}$	$1.3X10^{8}$
9	1	58.3	$1.0X10^{4}$	$1.4X10^{8}$
	2	36.9	$3.2X10^{5}$	$2.0X10^{8}$
	3	17.8	$1.0X10^{6}$	$6.4X10^{7}$
	4	23.2	$1.0X10^{5}$	$8.1X10^{7}$

*See footnotes a, b, c and d (Page 148, 149) for composition of media.

**Determined on a Fisher electrophotometer fitted with a 4250A filter.

TABLE III

Comparative estimates of numbers of pleuropneumonia-like organisms propagated in four different media.

Days incubation	Media*	Percent** transmission	Viable count/ML	Microscopic count/ML
2	1	77.2	$1.0X10^{6}$	$5.9X10^{7}$
	2	49.3	$3.2X10^{5}$	$1.0X10^{8}$
	3	25.7	$3.2X10^{3}$	$1.3X10^{8}$
	4	38.5	$1.0X10^{4}$	$6.8X10^{7}$
9	1	62.4	$1.0X10^{7}$	$6.5X10^{7}$
	2	48.2	$3.2X10^{2}$	$5.1X10^{7}$
	3	24.1	$1.0X10^{1}$	$3.8X10^{7}$
	4	37.7	$1.0X10^{2}$	$4.0X10^{7}$

*See footnotes a, b, c and d (Page 148, 149) for composition of media.

**Determined on a Fisher electrophotometer fitted with a 4250A filter.

DISCUSSION

Before counting PPLO, one must thoroughly familarize himself with the variations in morphology which may be present in a given culture under different conditions and with the artifacts which may be in the medium or the stain.

The Van Roekel avian PPLO strain was chosen as a suitable strain for counting because of its rather uniform appearance and its tendency not to clump. Strains of PPLO not meeting these requirements may prove more difficult to count accurately.

Concentrated specimens containing roughly 1,000 to 5,000 cells per oil immersion field were used for counting because it is difficult to distinguish PPLO from debris when only small numbers of PPLO are present. This is true because of the small size of the cells and because of their apparent inability to stain well when small numbers are stained.

The percent light transmission data obtained on this study must be regarded as unreliable in view of the relatively good correlation between viable counts and direct microscopic counts. These unreliable results are probably due to the different compositions of the media used. This is suggested by the fact that the more complicated media consistently produced much greater turbidities even though the PPLO cells were washed to prevent this. In view of these data, it appears that comparative growth studies on media of different composition cannot be accurately evaluated by the turbidimetric methods of measurement herein described.

The observation that the largest viable counts are within one log of the largest direct counts seems to indicate that these are reliable methods of estimating PPLO numbers. The discrepancies between the data obtained by these methods might indicate that phenomena such as the production of acid, growth stimulation by certain substances such as yeast hydrolysate, or temperature variation might cause PPLO cultures to enter their death phase earlier than normal. Also, small inocula such as those occurring in the higher viability determination dilutions may reduce the apparent number of viable cells in a given sample. These comparative data also suggests that PPLO cells die quickly under adverse conditions but autolyse slowly after death.

ACKNOWLEDGMENT

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SUMMARY

Avian pleuropneumonia-like organisms (PPLO) (Van Roekel strain) stained on glass slides have been counted directly by microscopic examination. The average difference from the mean count (two operators) for the two operators counting was 2.0 percent.

PPLO which had been cultivated on four different media were prepared for counting by centrifugation at 14,000 times gravity for 30 minutes at 6 C and resuspension in distilled water to one-tenth the original volume. The resuspended material was used to prepare Giemsa stained preparations for direct counting. Viable counts on homologous media and percent light transmission data (electrophotometer) were also obtained.

Direct microscopic count data and viable count data were in agreement except where conditions may have existed which could have caused the numbers of viable PPLO to be reduced. Data on percent light transmission were not in agreement with the other data; this may have been due to the composition of the media used.

REFERENCES

1. Holmes, B. E., and A. Pirie. Growth and metabolism of the bovine pleuropneumonia virus. Brit. Jour. Exp. Pathol., *13*, 364-370, 1932.
2. Keller, R., and H. E. Morton. The growth of pleuropneumonia-like organisms of human origin: Cultivation in the developing chick embryo and an in vitro growth cycle. Jour. Bact., *67*, 129-134, 1954.
3. Lecce, J. G., and H. E. Morton. Metabolic studies on three strains of pleuropneumonia-like organisms isolated from man. Jour. Bact. *67*, 62-68, 1954.
4. Liebermeister, K. Ein Nahrsubstrat zur Zuchtung von Organismen der Pleuropneumonia-(PPLO)-Gruppe. Z. Hyg. Infektionskrankh., *140*, 132-141, 1954.
5. Rodwell, A. W., and E. S. Rodwell. The breakdown of carbohydrates by *Asterococcus mycoides*, the organism of bovine pleuropneumonia. Australian Jour. Exp. Biol. Sci., *7*, 18-30, 1954.
6. Smith, P. F. Growth of pleuropneumonia-like organisms. Applied Microbiol., *4*, 254-259, 1956.
7. Smith, P. F., and H. E. Morton. The separation and characterization of the growth factor in serum and ascitic fluid which is required by certain pleuropneumonia-like organisms. Jour. Bact., *61*, 395-405, 1951.
8. Smith, P. F., and H. E. Morton. Nature of the growth inhibitor in some mammalian sera for pleuropneumonia-like organisms of human origin. Proc. Soc. Exp. Biol. Med., *83*, 65-69, 1953.

EXPERIMENTAL FUNGOUS INFECTIONS IN CHICKENS†

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THE increased economic value of the poultry industry has warranted research on fungous infections of chickens. Bullis[4] discussed the various types of fungous infections and referred to some early work on this subject.

An excellent article by Urbain and Guillot,[16] gives a complete history of Aspergillus infections in birds dating back as far as 1815. They reported that Perroncito in 1884 observed a miliary form of aspergillosis in a hen. They found that an intravenous spore inoculation in pigeons provided the most reliable means of experimental infection and stated that *Aspergillus fumigatus* and *A. flavus* were pathogenic. They were unable to prove that *A. glaucus* was pathogenic.

A high mortality due to respiratory fungous infections in turkeys has been reported in 1952 by Witter and Chute.[18]

Jordan[13] in England reported the incidence of fungi in the lungs of 25 chickens and found that *Penicillium spp.* was most prevalent with *Aspergillus fumigatus, A. glaucus, A. candidus, A. versicolor* and Mucor being found occasionally. The fungous flora of chickens with a respiratory disease has been studied by Chute *et al.*[8]

The research data presented here resulted from attempts

† The project was supported in part by funds from Lederle Laboratories, Pearl River, New York.

to determine which of the fungi isolated from chickens were pathogenic. Brooksbank and Austwick[3] have shown that there were different susceptibilities between pure and hybrid strains and some differences between several pure-bred lines of chickens. Their studies indicated hybrid chicks were much more resistant under identical environmental conditions. A similar resistance factor was demonstrated by Winner[17] who found that rabbits possessed a considerable degree of natural immunity to an intravenous injection of virulent *Candida albicans* and in some cases it was difficult to kill animals with massive doses.

The presence of the specific fungus, *A. fumigatus* (aspergillosis), was established in England in 18 free-living wild birds such as the herring gull, jackdaw and woodpigeon by McDiarmid.[14] Halloran[12] in her bibliography of references to diseases of wild birds, refers to fungous infections in the United States in the wood duck, wild geese, mallard duck, ruffed grouse, American coot and the snowy owl.

Chute *et al.*[7] published on the pathology of *A. fumigatus* in a flock of 5000 caponized cockerel chickens and made a brief review of the literature on the current mortality problem in domestic chickens.

Blaxland and Markson[2] studied the transmissibility and pathogenesis of *C. albicans* in turkey poults. Enteral and parenteral administration of a culture of *C. albicans* produced infection in most of the inoculated and in-contact poults and sub-clinical moniliasis in a few of them. A field survey indicated that *C. albicans* may be present in birds as a commensal and may even cause gross lesions in the crop without any clinical sign of disease.

Another purpose of the present study was to determine if any toxicity resulted from experimental infections. Many reports show that fungi have toxic properties. Zeller[19] reported from Germany that Aspergillus, Mucor and Penicillium species contaminating hay and straw were responsible for illness in horses. The illness was characterized by a gastrointestinal inflammation. Sippel *et al.*[15] reported 24 outbreaks of poisoning in pigs fed moldy maize. Cultures of *Aspergillus*

taumanii were obtained from the affected livers. Poisoning was also observed with a moldy maize in a herd of 48 cattle, nine of which died. Carll *et al.*[5,6] recovered a toxic substrate from *Aspergillus clavatus*. The organism was isolated from feed pellets and produced toxic symptoms in mice. They also found an ether extract of *A. fumigatus* was topically toxic to rabbits, calves and horses.

Forgacs and Carll[10] found that day-old White Rock chicks developed severe hemorrhages throughout the body when fed scratch grain on which selected strains of Aspergillus, Penicillium and Atlernaria species had been cultured.

The natural reservoir of many species of fungi is not necessarily the host itself but may be the soil or air. This point has been adequately established by Ajello[1] who isolated 21 pathogenic or potentially pathogenic fungi from non-living sources. Most of these isolates were from soil from several different parts of the world and included such fungi as *A. fumigatus, C. albicans, Coccidioides immitis, Cryptococcus neoformans, Histoplasma capsulatum* and *Trichophyton mentagrophytes*. Emmons[9] in a recent paper explained that the lungs of rodents were common carriers of saprophytes such as Myxotrichum and Gymnoascus as well as a pathogen *Haplosporangium parvum*.

There is a paucity of literature available on the pathogenicity and toxicity of fungi for chickens. Bullis[4] stated that aspergillosis could readily be produced by intrathoracic injection in chickens and pigeons but he did not relate any detailed information.

MATERIALS AND METHODS

The fungi used in the experiment had been isolated from birds showing a respiratory disease except in the few cases which are otherwise indicated. The fungi were grown in glass bottles (9 x 4.5 cm) with plastic screw caps with approximately one cm of Czapek's media in the bottom. The original cultures were transferred to these bottles and allowed to grow until a lush growth was present. Then to each bottle was added 6 ml of sterile distilled water containing Leconal,* a

* Laboratory Equipment Corporation, St. Joseph, Mich.

wetting agent. The purpose of the wetting agent was to ensure that as many spores as possible would be in suspension. Immediately after adding the water to the fungus culture and thoroughly mixing, the suspension of spores and some mycelia were drawn up in a syringe with a 20 gauge needle. The bird was then inoculated posterior to the last rib on the left side which would be in the region of the abdominal air sac. The inoculated dose was 0.5 ml in some cases and 1 ml in others (table 1).

Six White Rock chicks, four weeks old, were used for each culture. The last eight cultures were used on New Hampshire-Barred Plymouth Rock hybrid cockerels. Seven days after injection and every week thereafter one bird was removed from each group unless a bird died.

A complete necropsy was performed on every inoculated bird. After thoroughly wetting the feathers with soapy water the skin was removed and the abdominal cavity opened. A piece of the left abdominal air sac and left lung was aseptically removed and placed in a bottle of Czapek's media. The following tissues were then removed and placed in 10 percent formol-saline; trachea, lung, air sac, heart, spleen, liver, abdominal wall (from site of inoculation), kidney, intestine and femur. These tissues were sectioned and stained with hematoxylin and eosin and the lungs, air sacs as well as any other organs showing lesions were stained by the Gridley technique.[11]

The chicks were raised in isolation houses prior to inoculation and had not been exposed to any other disease agents. After the experimental inoculation they were wing-banded and reared together, in batteries. As they grew, they were placed in separate batteries. A commercial chick ration was fed. Two non-infected controls were always maintained with each experimental group.

RESULTS

Table 1 lists the organisms used and the resultant gross or microscopic infections. Clinically, very little change could be observed. The birds appeared to be healthy in spite of the fact that many of them had spores and mycelia in the tissues

TABLE I

Pathogenicity of fungi in chickens.

Culture	Inoculation dose	Necropsy time after infection	Gross (1) pathology	Micro (2) pathology	Culture Re-isolation
Paecilomyces varioti	0.5 ml.	1 week	—	2+	P. varioti
		3 weeks	C	1+	Suspicious (4)
		4 weeks	P	2+	Negative
Scopulariopsis brevicaulis	0.5 ml.	1 week	C H (liver)	1+	Negative
		3 weeks	—	1+	Negative
Penicillium citrinum	0.5 ml.	1 week	C	2+	Negative
		3 weeks	P, C	2+	Negative
Hormodendrum spp.	1.0 ml.	1 week	—	1+	Negative
Aspergillus flavus	1.0 ml.	1 week	P	—	Negative
		3 weeks	P	—	Negative
		4 weeks	C	2+	Negative
		5 weeks	—	+	Negative
Aspergillus niger	1.0 ml.	1 week	—	—	A. niger
		4 weeks	C	+	A. niger
Penicillium roqueforti	1.0 ml.	1 week	P	1+	Suspicious
		3 weeks	—	2+	Negative
		5 weeks	—	1+	Negative
Aspergillus clavatus	0.5 ml.	1 week	P (abd.wall)	—	Negative
		2 weeks	—	—	A. clavatus
		3 weeks	—	1+	Negative
		4 weeks	—	1+	Negative
Penicillium lilicenum	0.5 ml.	1 week	—	1+	Negative
		3 weeks	—	1+	Negative
		6 weeks	—	1+	Negative
Aspergillus candidus	1.0 ml.	1 week	P	1+	Negative
		4 weeks	—	1+	Negative
Penicillium puberlum	1.0 ml.	1 week	P	1+	Negative
Penicillium brevi-compactum	1.0 ml.	2 weeks	P	1+	Negative
		4 weeks	—	2+	Negative
		5 weeks	—	2+	Negative
Aspergillus glaucus near A. amstelodami	1.0 ml.	1 week	—	—	A. glaucus group
		2 weeks	—	2+	Negative
Aspergillus glaucus near A. chevaleri	1.0 ml.	1 week	—	1+	Suspicious
		6 weeks	—	1+	Negative
Aspergillus glaucus near A. ruber	1.0 ml.	3 weeks	—	1+	Suspicious
		5 weeks	—	2+	Negative
Trichoderma spp. near T. glaucus	1.0 ml.	1 week	P	2+	Suspicious
		5 weeks	—	—	Suspicious
Penicillium oxalicum	1.0 ml.	2 weeks	—	2+	Negative
		3 weeks	—	1+	Suspicious
		5 weeks	—	1+	Negative
Penicillium stoloniferum	1.0 ml.	2 weeks	—	1+	Negative

TABLE I

(Continued)

Trichophyton spp.	1.0 ml.	2 weeks	—	1+	Negative
		5 weeks	—	1+	Suspicious
A. fumigatus (eye) #1882	1.0 ml.	1 week	P	2+	A. fumigatus
		2 weeks	P	2+	A. fumigatus
		3 weeks	P	2+	A. fumigatus
		5 weeks	—	2+	A. fumigatus
		6 weeks	P	2+	A. fumigatus
A. fumigatus (3) (W. Va.) #1972	0.5 ml.	1 week	—	2+	A. fumigatus
		2 weeks	—	2+	Negative
		3 weeks	P	2+	Negative
		4 weeks	—	2+	Suspicious
A. fumigatus (3) (W. Va.) #1977	0.5 ml.	3 days	P	2+	A. fumigatus
		2 weeks	—	2+	Negative
		3 weeks	—	2+	Negative
Penicillium chrysogenum	1.0 ml.	3 weeks	P (liver, lungs)	1+	Negative
Alternaria spp.	0.5 ml.	1 week	—	2+	Negative
		4 weeks	—	2+	Suspicious
Penicillium islandicum	1.0 ml.	3 weeks	—	2+	Suspicious
		4 weeks	C	+	Negative
Aspergillus restrictus, near A. glaucus	1.0 ml.	1 week	—	1+	Negative
		4 weeks	—	1+	Negative
Trichoderma koningi	0.5 ml.	1 week	—	1+	Suspicious
		2 weeks	—	1+	Suspicious
		4 weeks	—	1+	Negative
		5 weeks	—	+	Negative
		6 weeks	—	2+	Negative
Stemphylium spp.	1.0 ml.	1 week	P	—	Suspicious
		2 weeks	—	2+	Suspicious
Penicillium near stoloniferum in PBC series	1.0 ml.	1 week	P	2+	Suspicious
		2 weeks	—	2+	Negative
		4 weeks	—	2+	Negative
Penicillium cyclopium	1.0 ml.	1 week	P	1+	Suspicious
		2 weeks	—	2+	Negative
		3 weeks	—	1+	Negative
Penicillium near Cyclopium	1.0 ml.	3 weeks	C	—	Negative
		4 weeks	P	1+	Negative
Penicillium terrestre series	1.0 ml.	4 weeks	—	1+	Negative
		5 weeks	—	2+	Negative
	1.0 ml.	3 weeks	—	1+	Negative
		4 weeks	—	1+	Negative
Near Chlamydomyces		5 weeks	—	1+	Negative

(1) GROSS PATHOLOGY
P—Plaques
C—Cloudy Air Sacs
H—Hemorrhage

(2) MICROSCOPIC PATHOLOGY
2—Mycelia and Spores
1—Spores
+—Granulomas

(3) Culture received from Dr. N. Olson, Morgantown, W. Virginia

(4) Mycilial growth but no sporulation for identification.

as demonstrated by histological methods. Occasionally some respiratory distress could be observed in birds injected with the most pathogenic strains of *Aspergillus fumigatus*.

The gross examination occasionally revealed cream colored plaques in the muscle wall at the site of injection, as well as in the air sacs and lungs. The left abdominal air sac and lung were carefully observed for such lesions. Experimental infection was easily accomplished with some strains such as *A. fumigatus* (table 1). In severe cases, an enlargement of the liver was noted, as well as plaques on the liver.

The histopathology revealed granulomas 7 days after experimental infection. The first reaction to injury was an extensive mononuclear infiltration at the site of injection which was quickly followed by a granulomatous inflammation. This was characterized by the presence of numerous granulomas which, in most cases, contained many "giant cells" or were completely surrounded by them (figure 1). The granulomas were composed of a necrotic center surrounded by epithelioid cells which coalesced to form "giant cells".

Several cases of myocarditis were characterized by acute inflammations with heterophils predominating (figure 2).

The spores in the tissue often appeared refractile when stained by either bematoxylin and eosin or Gridley. Granulomas formed readily as a reaction to the injury produced by spores (figure 1). This was observed in many cases. Spores remained intact in the tissues for as long as six weeks.

The mycelia were noted to take different forms with different genera of fungi. *Aspergillus fumigatus* and *Trichoderma spp.* near *T. glaucus* produced the most prolific type of mycelial growth (figure 3). This points out the advantage of using a special fungous stain for tissue studies. Some mycelia appeared to be degenerating and were flattened as was evidenced by *Penicillim brevi-compactum* (figure 4).

Figure 5 is a photo of a six-day-old chick from which a pathogenic strain of *A. fumigatus* was isolated. Tissue sections did not reveal any other disease and no lesions of aplastic anemia was present in the bone marrow of the femurs.

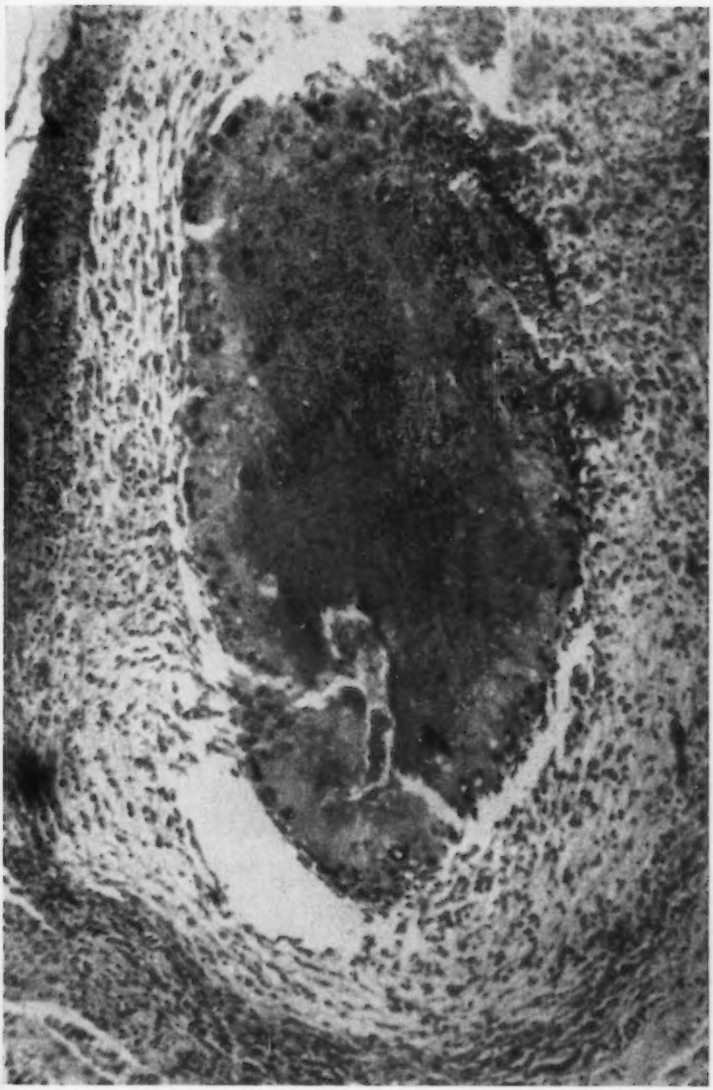

Fig. 1.—Muscle wall 14 days after injection. A granulomatous inflammatory process has developed. The granuloma is surrounded by giant cells. This bird was injected with *Penicillium citrinum*. Hematoxylin and eosin stain, × 100.

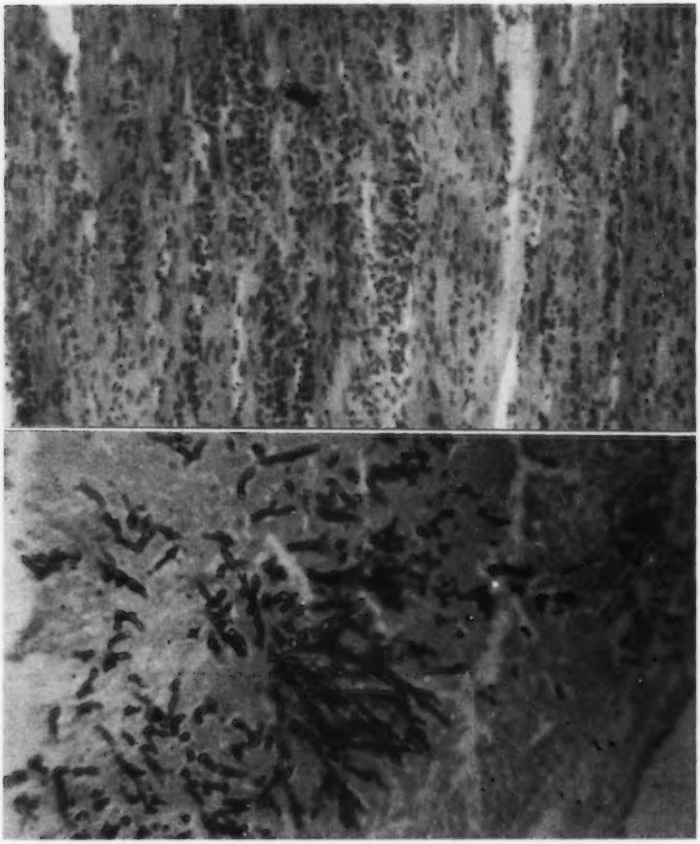

Fig. 2 (top)—Myocarditis produced by *Penicillium cyclopium* seven days after injection. Hematoxylin and eosin stain, × 555.

Fig. 3 (bottom)—Air sac of bird injected with *Trichoderma koningi*. Note the presence of mycelia and spores. Gridley stain, × 600.

DISCUSSION

It was difficult to decide why more isolations could not be made after experimental injection (table 1). It could have been that the reaction to injury produced a granuloma rapidly

Fig. 4.—Muscle wall 28 days after injection with *Penicillium brevi-compactum*. The mycelia have degenerated and appear flattened. Gridley stain, × 70.

and the spores were trapped in the granuloma upon culture or that mycelial growth did not continue in vitro to sporulation. There was no evidence of tissue reaction four weeks after experimental infection with most of the genera. It was assumed that the body defenses had controlled the experimental infection by this time. Exceptions were *A. fumigatus, Paecilomyces varioti, Penicillium brevi-compactum, A. glaucus, Trichoderma koningi, P. terrestre,* which had mycelia in the tissue four weeks or longer. Other than *A. fumigatus* and

Fig. 5.—Gross photo of infected chicken with the plaque which was removed from an eye. *Aspergillus fumigatus* was isolated. × 3.

T. koningi which produced good mycelia, the mycelia of the other genera of fungi appeared to have degenerated as in figure 4.

In this study some birds obviously received a much larger dose of spores than others since standardized inoculums were not prepared.

The merits of the Gridley stain can be noted from the photomicrographs. Granulomas are common in birds and a

special stain may be necessary to decide the exact etiological agent.

A great variation in pathogenicity exists between strains of the same organism. This feature has been observed in this laboratory with *A. fumigatus* (table 1). Some strains are innocuous whereas others are very pathogenic.

ACKNOWLEDGMENTS

The identification of original cultures was done by Dr. H. D. Tresner, Medicinal Chemical Research Section, Lederle Laboratories, Pearl River, New York.

The authors express their appreciation to Elizabeth Cyr for technical assistance.

CONCLUSIONS

1. Different strains of fungi, particularly Aspergilli, have different degrees of pathogencity.
2. Different fungi were observed to produce different mycelial morphology in the tissue.
3. Granulomas form rapidly as a reaction to injury from some species of spores in the air sacs and lungs.
4. Bone-marrow sections did not reveal any aplastic anemia in birds infected with any of the fungi studied.
5. The following cultures which were used in experimental infections revealed both spores and mycelia in the tissue: *Paecilomyces varioti, Penicillium roqueforti, Penicillium brevi-compactum, Aspergillus glaucus* groups, *Trichoderma spp., Trichoderma koningi, Penicillium oxalicum, Aspergillus fumigatus, Alternaria spp., Penicillium islandicium, Stemphylium spp.,* and *Penicillium cyclopium.*
6. The following cultures showed only spores in the tissue: *Scopulariopsis brevicaulis, Hormodendrum spp., Aspergillus niger, Aspergillus clavatus, Penicillium lilacenum, Aspergillus candidus, Penicillium stoloniferum, Trichophyton spp. ,Penicillium chrysogenum,* and *Aspergillus restrictus.*

Although a few spores were observed in the tissue for as long as six weeks, generally none remained after four weeks.

7. Air sac inoculation was found to be a rapid and efficient method of screening fungi for pathogenicity in birds.

8. *Aspergillus fumigatus* and *Trichoderma spp.*, near *T. glaucus* appeared to be the most pathogenic.

REFERENCES

1. Ajello, Libero. Soil as natural reservoir for human pathogenic fungi. Science, *123*, 876-879, 1956.
2. Blaxland, J. D., and L. M. Markson. Observations on the transmissibility and pathogenesis of moniliasis in turkey poults. Brit. Vet. Jour., *110*, 139-145, 1954.
3. Brookshank, N. H., and P. K. C. Austwick. Susceptibility of inbred and outbred chicks to aspergillosis. Brit. Vet. Jour., *3*, 64-67, 1955.
4. Bullis, K. L. Diseases of Poultry. Biester and Schwarte, 3rd Edition, The Iowa State College Press, 1952.
5. Carll, W. T., and J. Forgacs. The significance of fungi in hyperkeratosis. Military Surgeon, *115*, 187-193, 1954.
6. Carll, W. T., J. Forgacs, Albert S. Herring, and G. Bill Mahlandt. Toxicity of aspergillus fumigatus. Vet. Med., *50*, 210-212, 1955.
7. Chute, H. L., J. F. Witter, J. L. Rountree, and D. C. O'Meara. The pathology of a fungous infection associated with a caponizing injury. Jour. Am. Vet. Med. Assoc., *127*, 207-209, 1955.
8. Chute, H. L., D. C. O'Meara, H.D. Tresner, and E. Lacombe. The fungous flora of chickens with infections of the respiratory tract. Am. Jour. Vet. Res., *17*, 65, 763-765, 1956.
9. Emmons, Chester W. Isolation of myxotrichum and gymnoascus from the lungs of animals. Mycologia, *46*, 334-338, 1954.
10. Forgacs, J. and W. T. Carll. Preliminary mycotoxic studies on hemorrhagic disease in poultry. Vet. Med., *50*, 172, 1955.
11. Gridley, Mary F. A stain for fungi in tissue sections. Am. Jour. Clin. Path., *23*, 303-306, 1953.
12. Halloran, Patricia O'Connor. A bibliography of reference to diseases of wild mammals and birds. Am. Vet. Med. Assoc. Publication, *16*, 1955.
13. Jordan, F. T. W. The incidence of fungi in the lungs of poultry. British Vet. Jour., *110*, 25-26, 1954.
14. McDiarmid, A. Aspergillosis in freeliving wild birds. Jour. Comp. Path., and Therapeutics, *65*, 246-249, 1955.
15. Sippel, W. L., J. E. Burnside, and M. B. Atwood. A disease of swine and cattle caused by eating moldy corn. Proc. 90th Ann. Meet. Am. Vet. Med. Assoc., 174-181, 1953.
16. Urbain, A., and G. Guillot. Les aspergillosis aviaires. Rev. Path. Comp. et Hyg., *38*, 929-955, 1938.
17. Winner, H. I. Immunity in experimental moniliasis. Jour. Path. and Bact., *71*, 234-237, 1956.
18. Witter, J. F., and H. L. Chute. Aspergillosis in turkeys. Jour. Am. Vet. Med. Assoc, *121*, 387-388, 1952.
19. Zeller, R. Uber schimmelpilzvergiftungen bei haustieren. Berl. Munch. tierarztl, Wschr, *68*, 99-101, 1955.

FURTHER OBSERVATIONS CONCERNING ROUS VIRUS NEUTRALIZING ANTIBODIES AND NEOPLASIA. I. IN FARM FLOCKS

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ACCORDING to Winton,[10] Rous virus neutralizing antibodies (RVNA) have been observed by Duran-Reynals in the sera of chickens from the Regional Poultry Research Laboratory (RPL) with lymphomatosis. Forty-five percent of the sera from RPL Line 9 chicks hatched in Iowa were found to contain RVNA, while the parent stock experienced 41 percent lymphomatosis.[6]

Kenzy and Neuzil[7] found RVNA in more than 60 percent of the sera from farm flocks in Iowa with 30 to 50 percent losses associated with fowl lymphomatosis, while flocks with less than a 5 percent loss due to tumors had a zero to 10 percent occurrence of these antibodies.

These observations raise questions concerning the susceptibility of a chicken flock to more than one type of tumor agent. Would certain families and lines with a high susceptibility to the lymphoid tumor agent(s) also be susceptible to connective tissue tumor agents such as the Rous virus?

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Scientific paper #1556, Project No. 1163.

Olson[8] found six types of neoplasia in a flock where the total mortality was 57 percent. Kenzy and Neuzil[7] found four kinds of neoplasia in a flock with a history of 30 percent losses associated with lymphomatosis.

Since further information concerning common susceptibility to Rous and lymphoid tumor agents was desirable, a number of flocks in which tumors were a problem were studied. This report is concerned with these studies.

MATERIALS AND METHODS

History of Flocks.

Flock 1. This was a Brown Leghorn flock of 250 hens in which 45 percent died before 15 months of age. No unusual losses were reported until the hens began to lay at five to six months. At that time "range paralysis", "big livers", and six cases of large skin tumors were reported by the owner. Two of the four hens submitted for necropsy exhibited extensive lymphoid tumor involvement of the subcutis (figures 1 and 2). A myxoma of the subcutis near the distal portion of the tibiotarsus was found in the third chicken, and the fourth had lesions of visceral lymphomatosis.

A month later, 25 Brown Leghorn hens, two Bantams and nine White Leghorns from one laying house were examined. Skin tumors, varying in diameter from a few millimeters to a centimeter, were observed on eight of 25 Brown Leghorn hens. No skin tumors were observed on any of the Bantams or White Leghorns housed with the Brown Leghorns. Blood samples for the Rous Virus Neutralization Test (RVNT) were collected from some of these hens.

Flock 2. This flock consisted of three White Leghorn strains identified as A, B and C with about 500, 425 and 800 survivors in each at 12, 10 and 9 months of age, respectively. The different strains were held in laying houses about 35 feet apart. A combination wire and wooden partition separated different strains in the same building. The laying houses had been used continuously for 8 years without complete depopulation.

Losses associated with enlarged livers and leg paralysis were reported soon after the pullets were placed in the laying

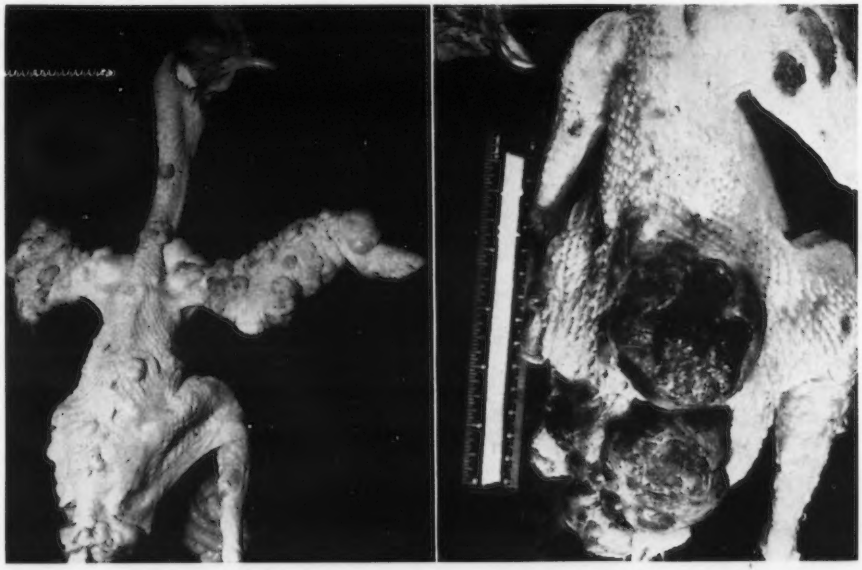

Fig. 1 (left) and 2 (right). Two hens with extensive lymphoid tumor involvement of the subcutis. Flock 1.

house at 5 months of age. Losses of 5, 20 and 10 percent for strains A, B and C, respectively, were reported by the owner as being associated with big livers and leg paralysis. Eight dead hens were necropsied on the farm. The enlarged livers in these hens were typical for fowl lymphomatosis. Blood samples from 5 to 10 percent of the survivor hens in each strain were collected for the RVNT.

Flock 3. This was a White Leghorn flock of 600 hens. Deaths occurred shortly after the birds were housed for laying at 6 months of age. At least 40 percent of them were lost within 4 months. Atrophy of the comb, decrease in weight and greatly enlarged livers were described by the owner and feedman. Two chickens submitted for necropsy had typical lesions of visceral lymphomatosis.

Technique for the Rous Virus Neutralization Test (RVNT).

The procedure originally described by Duran-Reynals[3] and modified by Kenzy[5] has been further adapted for demonstration of neutralizing antibodies.

Ten percent cell-free extracts of Rous tumor tissue were prepared in Simm's solution and stored in vials which were evacuated to 24 inches mercury for 3 minutes, frozen in an alcohol-dry ice mixture and then held in a dry ice chest (modification of Bryan's method[1]).

The Rous virus was titered in the usual manner,[5] employing seven chicks per dilution. Simm's solution, with 25 percent normal avian serum added, was used in preparing the dilutions of Rous virus for titration. The 50 percent infective dose (ID_{50}) was calculated according to the method of Reed and Muench.[9] One plus reactions were considered in the calculation of the ID_{50}.

The frozen virus was thawed and diluted with Simm's solution so that, when mixed with an equal volume of avian serum (diluted 1:2 with saline), about 10 to 30 ID_{50} were present in 0.2 ml of the serum-virus mixture. Serum-virus mixtures were held in a refrigerator at 5 C overnight before inoculation.

If a Rous tumor developed during a 30-day observation period in any of the three chicks inoculated with the serum-

virus mixture, neutralizing antibodies were not considered present in the test serum. Presence of neutralizing antibodies in the test serum was considered evidence of previous infection and susceptibility to the Rous virus or some antigenically related agent.

RESULTS

TABLE I

Summary of tumor losses and prevalence of Rous virus neutralizing antibodies.

Flock No.	Neoplasms observed	Percent mortality*	Positive for RVNA(a)	
1	Lymphocytoma; Myxoma	45	23/25[b]	92%
2—Strain A	Lymphocytoma	5	6/53	11%
2—Strain B	Lymphocytoma	20	33/60	55%
2—Strain C	Lymphoctytoma	10	33/58	57%
3	Lymphocytoma	40	19/43	44%

* More of the dead chickens from these flocks should have been necropsied by a poultry pathologist; however, these flock owners and the fieldmen for the breeders and feed companies were quite familiar with "big liver" disease.

[a]Neutralizing antibodies for the Rous virus.

[b]Numerator refers to number positive and denominator to number tested.

DISCUSSION

The number of hens in flock 1 (figures 1 and 2) with subcutaneous lymphoid tumors is unusual. Some workers[2,4] believe that mites may be associated with the transmission of tumor agents in such outbreaks.

The large percentage of sera which contained RVNA certainly suggests a high level of susceptibility to Rous virus or a Rous-like agent. Likewise, the occurrence of lymphoid tumors indicates a definite susceptibility to the lymphoid agent(s). It is interesting to note that Strain A from flock 2 had a low level of RVNA and likewise experienced a minimal loss associated with tumors.

A common susceptibility to both connective tissue and lymphoid tumor agents would help a breeder recognize lymphoid tumor resistant and susceptible lines or families. The advantage of a test which can be carried out in 30 days as compared with the long incubation period for lymphoid tumors is quite obvious. Carefully controlled studies of a

common susceptibility to Rous and lymphoid agents should be carried out in properly exposed populations.

ACKNOWLEDGMENTS

The photographs were made available through the courtesy of the Washington State College Photo Department, Office of Publication and Dr. G. R. Spencer, Department of Veterinary Pathology.

SUMMARY

1. Farm flocks experiencing severe losses associated with lymphoid tumors also appeared quite susceptible to the Rous agent on the basis of the Rous virus neutralization test.

2. The possibility of utilizing the Rous neutralizing antibody test for the recognition of lymphoid-resistant and susceptible lines is discussed.

3. A subcutaneous tumor outbreak of unusually high incidence is described.

REFERENCES

1. Bryan, W. R., J. B. Maloney, and D. Calnan. Stable standard preparations of the Rous sarcoma virus preserved by freezing and storage at low temperatures. Jour. Nat. Cancer. Inst., *15*, 315, 1954.
2. Carr, J. G. Towards the elimination of the fowl leukosis complex. Experientia, *9*, 326, 1953.
3. Duran-Reynals, F. Information concerning the technique of the Rous virus serum neutralization test. Yale University School of Medicine, New Haven, Conn. Personal communication, 1949.
4. Johnson, E. P. Transmission of fowl leukosis. Poultry Sci., *16*, 255, 1937.
5. Kenzy, S. G. Studies in avian neoplasia. I. A quantitative evaluation of neutralizing antibodies for the Rous sarcoma virus in avian serums. Am. Jour. Vet. Res., *13*, 388, 1952.
6. Kenzy, S. G. Studies in avian neoplasia. IV. Natural transmission of tumor agents through the egg. Am. Jour. Vet. Res., *14*, 455, 1956.
7. Kenzy, S. G. and P. V. Neuzil. Studies in avian neoplasia. II. Incidence of Rous virus-neutralizing antibodies in serums collected from flocks experiencing losses due to lymphomatosis. Am. Jour. Vet. Res., *14*, 123, 1953.
8. Olson, Carl, Jr. A study of neoplastic diseases in a flock of chickens. Am. Jour. Vet. Res., *3*, 111, 1942.
9. Reed, L. J. and H. Muench. A simple method of estimating fifty per cent endpoints. Amer. Jour. Hyg., *27*, 493, 1938.
10. Winton, B. Eleventh annual report of the Regional Poultry Research laboratory, *9*, 1950.

TRANSMISSION AND CONTROL OF INFECTIOUS SINUSITIS IN TURKEYS*

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AFTER two, consecutive, yearly outbreaks of infectious sinusitis and aerosaccitis among the Clemson College turkey flocks, a study was made to determine possible egg transmission of the infectious agent. Although various pathogenic organisms may cause infection in different areas of the respiratory tract of poultry, pleuropneumonia-like (PPLO) organisms are considered to be the etiological agents in the majority of cases where these infections exist. Isolation and cultivation of PPLO from turkey sinusitis material have been accomplished by several investigators.[2,3,4,5,7] However, the extent to which turkey sinusitis and aerosaccitis are egg transmitted is not completely known. Recent data by Hofstad[6] indicate that hens of turkey breeding flocks showing clinical signs of these two diseases are capable of transmitting the causative organisms by way of the egg. The purpose of this paper is to report the isolation of the turkey sinusitis agent from dead turkey embryos and hatched poults of an infected breeder flock and to present the results of a follow up control program among 130 severely infected young progeny.

* Tech. Contribution number 275, South Carolina Agricultural Experiment Station. Published by permission of the Director.

MATERIALS AND METHODS

Isolation media used throughout this experiment include Bacto-PPLO broth without crystal violet, Bacto-PPLO agar, and 7-day embryonating chicken eggs. The artificial media was prepared in the usual manner and enriched just before use with 2 percent serum fraction (Difco). Thallium acetate was added to the media used for primary isolations before autoclaving in the amount of 0.5 ml of a 1 percent solution per 100 ml of media. When making blind passages or inoculating embryonating eggs, thallium acetate was omitted from the broth. Penicillin was added to the PPLO broth and agar in concentration of 1,000 units per milliliter after autoclaving. The final reaction of the medium was pH 7.3. The carbohydrate media used in this study consisted of phenol red broth base (Difco) plus 2 percent serum fraction and 100,000 units of penicillin per 1000 ml of broth.

A portion of trachea and lungs of 50 pipped but dead turkey embryos and 25 culled day-old poults from an infected breeder flock was cultured for infectious sinusitis (PPLO). Individual specimens (trachea and lung) were placed in separate cotton stoppered tubes containing 5 ml of PPLO broth and incubated for 4 days at 37 C. At the end of this incubation period 0.1 ml was transferred to a second tube of PPLO broth (without thallium acetate) and incubated for 3 days at 37 C. One tenth mililiter from the preceeding culture tube was added to 3 ml of PPLO maltose broth and 0.1 ml allowed to flow evenly over the surface of a freshly prepared PPLO agar plate. Further isolation and identification follow the procedures of Grumbles *et al.*[5] and Adler *et al.*[1] When a suspected PPLO isolate was obtained (typical growth on agar and maltose fermentation) the usual series of carbohydrates were inoculated (dextrose, saccharose, lactose, maltose and mannite). Typical carbohydrate reactions of PPLO from turkey sinusitis infection is generally accepted as the fermentation of maltose, dextrose and saccharose but not lactose or mannite. Also 7-day-old embryonating chicken eggs and young turkeys were used for identification and pathogenicity studies. Six embryos were inoculated into the yolk sac in 0.2 ml amounts with PPLO or

maltose broth which had incubated for 72 hours at 37 C. Inoculated embryos were examined each day to determine the time of death. The yolk sac and amnioallantoic material were then collected aseptically, examined bacteriologically for the presence of PPLO, and check for possible bacterial contaminants. The young turkeys were inoculated with 0.5 to 0.7 ml of yolk sac-amnioallantoic material or broth into the infraorbital sinus. Cross infection of the inoculated birds was prevented by isolation and management.

Three weeks after hatching and while being brooded under conditions, a group of 300 poults from eggs laid by the sinusitis infected breeding flock experienced a definite outbreak of this disease. Thirty poults had swollen sinuses with typical, gelatinous exudate visible at the nostril. Approximately 100 had clinical symptoms of aerosaccitis with persistent cough, rales, and difficult breathing. The only stress factor which might have influenced this outbreak was intranasal Newcastle disease vaccination of the poults at one day of age. Several hundred other poults on the same premises and from the same breeding flock were vaccinated in a similar manner but remained free of the disease.

All birds with one or both sinuses affected were injected with 50 ml streptomycin-dihydrostreptomycin sulfate directly into each swollen sinus. Aspiration of exudate from the swollen area was performed on those birds having impaired vision. Also, each bird with clinical evidence of aerosaccitis was given 100 mg of the above antibiotic intramuscularly. The minimum amount of antibiotic given an infected bird was 50 mg and the maximum 200 mg. When the birds were 8 weeks old the flock was divided into six groups, two of which were confined and the rest placed on range. The six groups were considered as one flock and the division in no way altered the results of the project.

RESULTS

Isolation of PPLO from the pipped but dead embryos or from the culled day-old poults was accomplished in five instances. Three isolates originated from 50 of the dead embryos examined. Isolations were obtained from the 22nd,

34th and 41st embryos. Two isolates originated from 25 culled day-old poults examined. These were handled and designated in the same manner as the dead embryos. The criterion of isolation included growth of the culture on PPLO agar, typical staining according to the method of Dienes and fermentation of maltose broth. The strains isolated also fermented dextrose and saccharose but not lactose or mannite. Subsequent passage in PPLO broth or the respective carbohydrate resulted in enhanced fermentation of the three fermentable sugars. Maltose broth on first culture always showed change to acid earlier than dextrose or lactose, the time varying from several hours to several days.

Inoculation of embryonating chicken eggs and young turkeys revealed some variation in the pathogenic properties of the strains isolated. Each isolate killed embryos according to a typical mortality pattern. This pattern showed initial deaths starting at 4 days post inoculation and extending up to the time of hatching. Both of the strains isolated from the culled day-old poults induced sinusitis in normal poults after inoculation with yolk sac-amnioallantoic material or broth. However, only one of the three strains originating from the pipped but dead turkey embryos were pathogenic after inoculation into the infraorbital sinus of four young turkeys. Development of sinusitis infection occurred 6 to 15 days after inoculation.

As shown in table 1, 113 cases of infectious sinusitis (upper form) developed in this flock of poults. This outbreak started at 3 weeks of age and, although treatment was initiated promptly, the infection was not completely eradicated during a 20 week medication period. The different groups were treated at 4-week intervals and each bird was examined twice weekly for recovery from infection.

Complete recovery after one treatment with the antibiotics occurred in one instance, group E which contained four birds. Recovery among the other groups after initial treatment was variable, 50 to 80 percent. The large number of infected birds in group D responded quite satisfactorily to initial treatment in that 75 percent recovered and remained free of infection during an observation period of 28 days. In

TABLE I

Results of antibiotic treatment of young turkeys with infectious sinusitis

Group	Age	Description	Date First Treatment*	Number and percent recovered** (cumulative totals) 6/1	6/29	7/27	8/24	9/21
A	3 wks.	First 30 birds with sinusitis. 100 others had lower respiratory distress. Acute type infection.	5/4	(15)50%	(22)73%	(25)83%	(25)83%	(27)90%
B	7 wks.	Twelve new sinusitis cases observed. 20 showing respiratory distress.	6/1	—	(8)66%	(10)83%	(12)100%	(12)100%
C	11 wks.	Five new cases observed during the third 4 week period. Lower respiratory infection chronic in 15 birds.	6/29	—	—	(4)80%	(3)60% ***	(5)100%
D	15 wks.	Sixty-two cases in acute form noticed during fifth 4 week period.	7/27	—	—	—	(47)75%	(50)80%
E	19 wks.	Four new cases noticed. Lower respiratory trouble still present in 8 birds.	8/24	—	—	—	—	(4)100%

50 mg. streptomycin-dihydrostreptomycin per infected sinus.
* Birds without sinus swelling but showing respiratory distress given 100 mg intramuscularly.
** Birds not recovered were treated at four week intervals on dates shown.
*** Relapse or reinfection.

view of the fact that the birds under observation and treatment were on range, no attempt was made to isolate injected individuals.

The 24 week average weight of the sinusitis infected and non-infected birds is presented in table 2. It may be noted that there was little or no difference in average weight between the different groups; the infected bronze birds were slightly lighter in weight than the non-infected bronze group while the opposite result held true for the white turkeys.

TABLE II

Average weight in pounds of sinusitis infected and non-infected Turkeys at 24 weeks of age

Bronze turkeys				White turkeys			
Infected		Non-infected		Infected		Non-infected	
Male	Female	Male	Female	Male	Female	Male	Female
21.0	13.6	21.4	13.8	15.9	9.2	15.6	8.9

DISCUSSION

The data add to our present knowledge that the turkey sinusitis agent is transmitted by way of the egg. Previous yearly outbreaks among turkeys in a closed flock suggested such transmission. Pathogenic PPLO were isolated from dead in shell and hatched poults from clinically infected hens. Further evidence of such egg transmission was observed when a severe outbreak of both infectious sinusitis and aerosacculitis occurred in a flock of 3-week-old poults. These poults were hatched from eggs produced by the infected hens mentioned above.

General environmental conditions or specific stress factors appear to be important in a natural outbreak of the various forms of the disease. Just what these conditions and factors are is not completely understood. It is difficult to believe that intranasal Newcastle disease vaccination of the poults alone at one day of age "triggered" this outbreak. Birds from other hatches of the same breeder flock and under similar management suffered no similar disorder.

Treatment of turkey sinusitis by individual injection of the swollen sinus is laborious and not always completely successful. The amount of streptomycin-dihydrostreptomycin sul-

fate used in this experiment and at intervals shown cannot be considered exceptionally effective. The monthly intervals for treatment were necessary because this flock was on a grazing experiment and each bird was handled on the dates shown. There was no real difference in weight gain between the infected and non-infectd birds, although in a few cases, further complication to aerosaccitis resulted in general debilitation and mortality.

SUMMARY

Pathogenic pleuropneumonia-like organisms were isolated from dead in shell and culled poults produced by sinusitis-infected turkey hens. In 300 of the hatched poults, an outbreak of sinusitis and lower respiratory tract infection occurred at 3 weeks of age. Treatment of 113 sinusitis infected birds with streptomycin-dihydrostreptomycin sulfate gave variable results during an observation period of 20 weeks. Infected and treated birds averaged approximately the same weight as non-infected stock at the termination of the experiment.

REFERENCES

1. Adler, H. E., R. Yamamoto, and R. A. Bankowski. A preliminary report of efficiency of various mediums for isolation of pleuropneumonia-like organisms from exudate of birds with chronic respiratory disease. Am. Jour. Vet. Res., *15*, 463, 1954.
2. Fahey, J. E. A hemagglutination inhibition test for infectious sinusitis of turkeys. Proc. Soc. Exper. Biol. and Med., *86*, 38, 1954.
3. Gianforte, E. M., E. L. Jungherr, and R E. Jacobs. A serological analysis of seven strains of pleuropneumonia-like organisms from air sac infection in poultry. Poul. Sci., *32*, 662, 1955.
4. Gross, W. B., and E. P. Johnson. Effect of drugs on the agents causing infectious sinusitis of turkeys and chronic respiratory disease (air-sac disease) of chickens. Poul. Sci., *32*, 260, 1953.
5. Grumbles, L. C., E. Phillips, W. A. Boney, Jr., and J. P. Delaplane. Culture and biochemical characteristics of the agent causing infectious sinusitis of turkeys and chronic respiratory disease of chickens. Southwest Vet., *6*, 166, 1953.
6. Hofstad, M. S. Egg transmission of infectious sinusitis of turkeys. Avian Diseases, *1*, 165, 1957.
7. Jerstad, A. C., C. M. Hamilton, and V. E. Smith. Preliminary report of probable egg transmission of infectious sinusitis in turkeys. Vet. Med., *44*, 272, 1949.

THE EFFECT OF ANTIBIOTICS ON THE INFECTIOUS SINUSITIS AGENT OF TURKEYS: PART I. EGG TRANSMISSION

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JERSTAD, Hamilton and Smith[6] in 1949 suggested that the infectious sinusitis agent might be egg transmitted when they isolated the agent from fresh turkey eggs. This work has since been confirmed at the University of Minnesota[7]. The purpose of this experiment was to determine if high level antibiotic therapy given to naturally infected hens in egg production would prevent egg transmission of the infectious sinusitis agent. The amount of pure antibiotics given each bird was purposely placed at a high level in the hope that we would on subsequent experiments be able to utilize a lower level of antibiotic which would also be effective.

MATERIALS AND METHODS

A natural outbreak of infectious sinusitis involving 100 female and 16 male turkeys was studied. These birds were purchased in the fall of 1955 from a closed breeding flock

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The tetracycline and chlortetracycline antibiotics were supplied by Lederle Laboratories. Erythromycin was supplied by Abbott Laboratories and the oxytetracycline was supplied by Chas. Pfizer and Co. Inc.

in which no infectious sinusitis had ever occurred. The birds on arrival were housed in one large group while pens were being constructed. The flock was tested for PPLO antibody by the rapid plate test with two commercial antigens*, and one obtained from Dr. H. E. Adler of the University of California. Only one bird was positive to the tests and we planned to eliminate this bird from the experiment. Shortly thereafter, a severe outbreak of infectious sinusitis occurred within the flock. The morbidity of the outbreak was almost 100 percent. Almost all of the birds developed rales and dyspnoea and in addition 54 percent of the birds developed enlarged infraorbital sinuses. The birds were tested for ornithosis by the indirect complement fixation test. However, the titers were not significant nor was there a significant rise in titers on subsequent bleedings. The birds were tested for Newcastle disease using the beta procedure of the hemagglutination-inhibition test (HI). These HI titers were also negative. Birds were sacrificed and tissues collected for isolation of the agent. Tracheas, lungs and air sacs were triturated and inoculated into 7-day-embryonated chicken eggs via the yolk-sac route. Twelve isolations of pleuropneumonia-like organism were obtained. These isolates were also cultured in semi-synthetic media and met the requirements of this group of organisms. Attempts to isolate the Fahey and Crawley agent[1,2] with a technique embodying filtration and antibiotics were unfruitful.

Selection and treatment. The infected birds were randomly selected and divided into nine groups, of ten hens and one tom. The birds were brought into production by artificial illumination and were trap nested. All birds served as control pens until 50 percent production was reached. The birds were then placed on antibiotics according to the following schedule. This therapy was continued for approximately five months.

Infertile eggs. Infertile eggs were sanitized in a 1:1000 solution of bichloride of mercury. An opening was made just over the air cell and the contents aseptically transferred to a 500 ml wide mouth erylenmeyer flask containing 100 ml of

* Lederle Laboratories and Salsbury Laboratories.

TABLE I

The treatment of breeding turkeys with various antibiotics. All antibiotics administered intramuscularly (IM) and intravenously (IV) were given every 48 hours for three injections and then once weekly thereafter.

Pen no.	Antibiotic in feed	Antibiotic injected
A	Control	Control
B	TC 1000 gm/ton feed	DS 50 mg/lb 1M
C	TC 1000 gm/ton feed	None
D	None	CTC 5 mg/lb IV
E	CTC 1000 gm/ton feed	CTC 5 mg/lb IV
F	None	OTC 5 mg/lb IV
G	OTC 1000 gm/ton feed	OTC 5 mg/lb IV
H	None	DS 50 mg/lb IV
I	EM 1000 gm/ton feed	EM 50 mg/lb IV

TC — Tetracycline (HCL)
CTC — Chlortetracycline (HCL)
OTC — Oxytetracycline (HCL)
DS — Dihydrostreptomycin (Sulfate)
EM — Erythromycin (Lactobionate)

the following media adjusted to pH 7.8.

Phenol red maltose broth (Difco) 21 Gm/L
Thallium acetate .5 Gm/L
Yeast hydrolysate 10 Gm/L*
Penicillin G 500,000 IU/L
PPLO extract (Difco) 2%

The flask was incubated at 37 C for four days and a 1 ml portion transferred to 20 ml of tubed media similar to that above with the exception that no carbohydrate was present. Samples were then passed serially in PPLO broth, and carbohydrate media for identification.

Dead and pipped eggs. The dead eggs were examined in a manner similar to that of the infertile eggs. The lesions were noted in the late embryos.

Hatched poults. A random number of poults were selected and destroyed at various ages and the lesions present were noted. Agent isolation was also attempted.

All data were correlated on "Unisort Punch Cards."†

* Nutritional Biochemicals, Cleveland, Ohio.
† Charles R. Hadley Co., Los Angeles, California.

RESULTS

Poults, pipped and late embryos from the infected hens on antibiotic therapy were examined for "air sac" lesions. Lesions were found in all groups. The greatest number of lesions were found in embryos and poults from the control group. The results are summarized in table 2.

TABLE II

Chi-square test of significance between control and treated groups

Pen	With lesions	Without lesions	Total	Percent reduction	E	(O - E)
A	95	410	505	0	63.38	31.62
B	45	281	326	26.63	40.91	4.09
C	50	309	359	25.94	45.05	4.95
D	32	270	302	43.65	37.90	—5.90
E	20	271	291	63.48	36.52	—16.52
F	35	282	317	41.31	39.78	—4.78
G	20	212	232	54.17	29.12	—9.12
H	35	227	262	28.97	32.88	+2.12
I	30	361	291	45.19	36.52	—6.52
Total	362	2623	2885			

Percent with lesions 12.55

$X^2 = \frac{(O - E)^2}{E} = 29.80$ $\quad P = .001$ for X^2_8

In table 2, the number of birds with lesions in the pens where antibiotics were given differs from the number of untreated birds with lesions. A chi-squared value of 29.8 was obtained which exceeds the chi-squared value for eight degrees of freedom with probability equal to .001 (expected to occur one time in 1000 due to chance alone). Hence the proportion of birds showing lesions differ significantly from pen to pen, presumably due to treatment of the dams.

Infertile eggs, poults pipped and late embryos from the infected hens were cultured for PPLO. The agent was not isolated from infertile eggs, dead embryos or pipped embryos; however, it was isolated from hatched poults of all pens. Some of these isolates were atypical. They multiplied and met

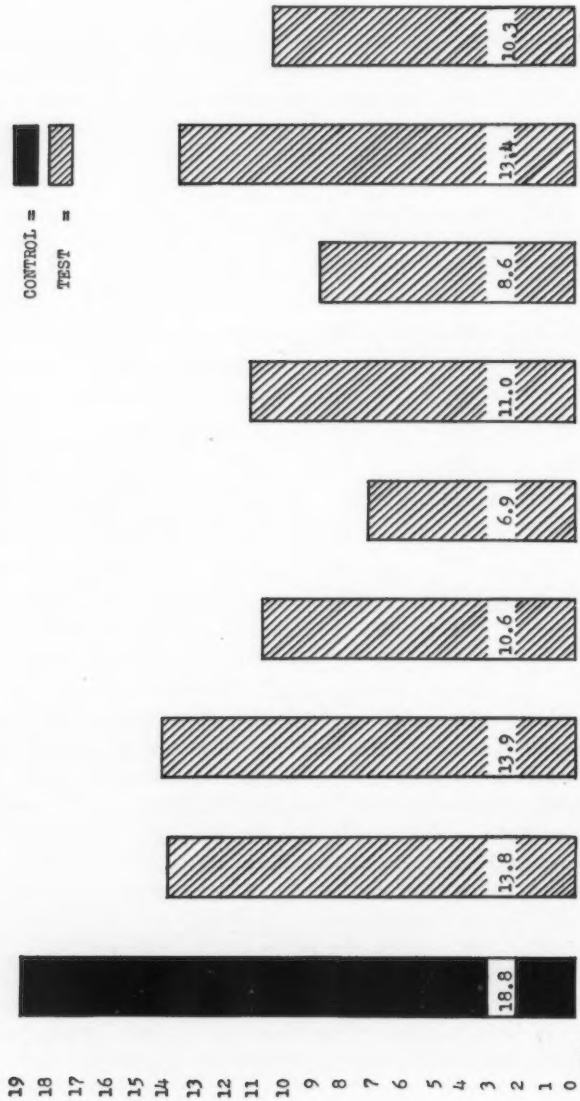

Fig. 1. Incidence of air sac lesions in poults and late embryos from dams on antibiotic therapy.

the requirements of PPLO morphologically and biochemically in semi-synthetic media but did not kill 7-day-embryonated chicken eggs.

Hatched poults were brooded and observed over a period of 5 months. No clinical sinusitis was observed in the poults. A random sample of the poults were stressed with Newcastle disease virus, bluecomb disease agent and Crawley virus. No infectious sinusitis was induced by these agents. The poults. were, however, found to be carriers of the pleuropneumonia-like agent.

DISCUSSION

The antibiotics used in this experiment have been observed by other workers to be quite effective against the pleuropneumonia-like organism of infectious sinusitis[3,4,5,9,10]. The authors expected that high level antibiotic therapy would prevent egg transmission of the infectious sinusitis agent. We were anxious to determine why egg transmission continued to occur during the antibiotic therapy. We then postulated that the antibiotic therapy may have selected antibiotic resistant strains of PPLO which were egg transmitted. In accord with this hypothesis antibiotic resistant strains of PPLO were recovered from the adult hens of the experiment after the prolonged high level antibiotic therapy[8].

Although no particular antibiotic therapy completely blocked egg transmission of the agent, an over-all benefit of antibiotic therapy was demonstrated by the results in favor of significant with a probability equal to .001 (table 2). In figure 1, it is observed that the lowest incidence of lesions was found in the progeny of the dams which were treated with chlortetracycline or oxytetracycline injected and also in the feed. (pens E and G). The next most effective antibiotic was erythromycin (injected and in the feed pen I). This was closely followed in effectiveness by chlorotetracycline or oxytetracycline injected only (pens D and F). The least effective antibiotic schedules were found to be dihydrostreptomycin injected plus tetracycline or either used alone (pens B, C and H).

ACKNOWLEDGMENTS

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SUMMARY

Prolonged high level antibiotic therapy of turkey hens did not prevent egg transmission of the infectious sinusitis agent to their progeny. The progeny were found to have "air-sac" lesions and were found to be carriers of the pleuropneumonia-like organism. A statistically significant reduction of lesions was observed in the poults and late embryos derived from the dams which were given antibiotic therapy. No clinically observable infectious sinusitis could be produced in the progeny by stressing the poults with viral agents.

REFERENCES

1. Fahey, J. E. A virus in chronic respiratory disease of turkeys. Nature, *177*, 90-91, 1956.
2. Fahey, J. E., and J. F. Crawley. Studies on chronic respiratory disease of chickens, II. Isolation of virus. Canad. Jour. Comp. Med., *18*, 13-21, 1954.
3. Gross, W. B., and E. P. Johnson. Effect of drugs on the agents causing infectious sinusitis of turkeys and chronic respiratory disease. Poultry Science, *32*, 260-263, 1953.
4. Hitchner, S. B. Streptomycin as a treatment for infectious sinusitis of turkeys. Poultry Science, *28*, 627-629, 1949.
5. Hoyt, H. H., B. S. Pomeroy, and M. H. Roepke. Susceptibility of the causative agent of infectious sinusitis of turkeys to sulfamethazine, crystal violet, and antibiotics. Proceedings Book, Am. Vet. Med. Assoc., 267-270, 1952.
6. Jerstad, A. C., C. M. Hamilton, and V. E. Smith. Preliminary report of probable egg transmission of infectious sinusitis of turkeys. Vet. Med., *54*, 272-273, 1949.
7. Mataney, C. F., B. S. Pomeroy, and O. H. Osborn. Studies on egg transmission of the agent of infectious sinusitis of turkeys. Proceedings Book, Am. Vet. Med. Assoc., 310-314, 1955.
8. Mataney, C. F. Studies on the agent of infectious sinusitis of turkeys. Thesis, Univ. of Minn., 1957.
9. Wong, S. C., and C. G. James. The susceptibility of the agents of chronic resipiratory disease of chickens and infectious sinusitis of turkeys to various antibiotics. Poultry Science, *32*, 589-593, 1953.
10. Yamamoto, R. The effect of certain antibiotics and chemical agents upon pleuropneumonia-like organisms of avian origin. Thesis, Univ. Calif., 1955.

THE ACUTE TOXICITY OF PROCAINE PENICILLIN G AND OF DIHYDROSTREPTOMYCIN SULFATE IN THE PIGEON AND THE CHICKEN

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DURING the past few years, penicillin and streptomycin preparations have been used rather widely by commercial and exhibition poultry flock owners in an effort to alleviate the symptoms of chronic respiratory disease in their stock. Occasionally, reports are obtained from flock owners that signs of acute toxicosis were observed following the intramuscular injection of a combined antibiotic preparation of this type. The present study was stimulated by such an experience in a flock of 43 racing homer pigeons. In support of a presumptive diagnosis, which was later confirmed by histopathological examinations, of chronic respiratory disease (CRD) in this flock, each pigeon was injected in the breast muscle with 0.2 cc of Dihydrocillin, readimixed*. This volume contained 40,000 units of procaine penicillin G and 50 mg of dihydrostreptomycin sulfate. It was subsequently reported that this dosage produced brief emesis in all pigeons in one-half to one hour after injection but that the entire flock recovered without additional signs of toxicosis. The above dosage was based upon a recommendation of Crawley and Fahey[1] for CRD in chickens. Their report suggested 150 to 200 mg of

* Products of the Upjohn Company, Kalamazoo, Michigan.

streptomycin plus 150,000 units of penicillin per bird which was assumed to be a four to five pound White Leghorn hen.

In the studies reported here, an attempt has been made to characterize the acute toxicological pattern of procaine penicillin G and dihydrostreptomycin sulfate in the pigeon and the chicken, and to establish the intramuscular LD_{50} values of these antibiotics for these two species.

MATERIALS AND METHODS

Thirty-one mature, common pigeons which weighed from 350 to 400 grams were identified by individual feather patterns and were housed in a wire-floored, chicken, growing battery. They received commercial pigeon grain ration, grit and water *ad libitum*. Immature White Leghorn chickens, mostly females which weighed about 600 grams, were housed similarly to the pigeons and received an all mash growing diet, grit and water *ad libitum*.

Individual birds of both of these species were injected intramuscularly with aqueous preparations of dihydrostreptomycin sulfate*, procaine penicillin G (Diurnal-Penicillin 300 M*) and a combination of these two antibiotics (Dihydrocillin*) as reported in the results and in tables I to IV. Two-thirds of the pigeons used in this study received more than one injection of both dihydrostreptomycin sulfate and procaine penicillin G following suitable rest periods during the cross-over studies of Experiments I and II. Postmortem examinations were made of all pigeons and chickens used in these studies.

RESULTS

Experiment I: In an attempt to evaluate the toxic effects in pigeons of procaine penicillin G and dihydrostreptomycin in the field dosage, a double cross-over study was done.

Group A: Ten pigeons which averaged 360 grams each were injected intramuscularly with 40,000 units of procaine penicillin G. Three of these pigeons vomited one to several times during a period of 40 to 90 minutes postinjection. No depression of activity was observed. All birds appeared normal two hours after injection.

* Products of the Upjohn Company, Kalamazoo, Michigan.

At three and one-half hours after the first injection, this group of pigeons received an intramuscular injection of 50 mg of dihydrostreptomycin sulfate. This latter injection produced profuse emesis in nine of the ten pigeons in ten to thirty minutes. In addition, the group showed somnolence and three pigeons became slightly ataxic (inability to perch, unsteadiness on legs). No symptoms were observed the following morning approximately 15 hours later.

Group B: Ten pigeons of comparable weight to those in group A were injected similarly with the same two antibiotics but in reverse order, i.e., dihydrostreptomycin followed by procaine penicillin G three and one-half hours later.

As in group A, a 50 mg injection of dihydrostreptomycin resulted in noticeable depression in all pigeons in group B in 30 minutes. Most of these pigeons assumed a drowsy attitude with partially closed eyelids. Two pigeons showed moderate ataxia which was characterized by unsteadiness of the legs and droop of the neck and wings. These birds assumed a posture of mild prostration and the tail was used as a prop (figure 1). The vomiting reflex was observed in one pigeon. These symptoms were not present after one hour postinjection at which time the latter two pigeons were able again to perch. The injection of procaine penicillin G induced emesis in seven of the group B pigeons in 15 to 40 minutes. No symptoms were observed 15 hours later.

Experiment II: Three days later experiment I was repeated with Group A and B pigeons. The two injections which each pigeon received were reversed from the order of the respective groups in experiment I. The interval between injections was extended to 48 hours.

Group A: Following the intramuscular injection of 50 mg of dihydrostreptomycin, these ten pigeons became somnolent. Two of the three pigeons of this group which showed ataxia in experiment I again were affected similarly. Three other pigeons vomited in 35 to 60 minutes postinjection. The injection of procaine penicillin G two days later resulted in emesis in one of the ten pigeons.

Group B: The procaine penicillin G injection produced emesis

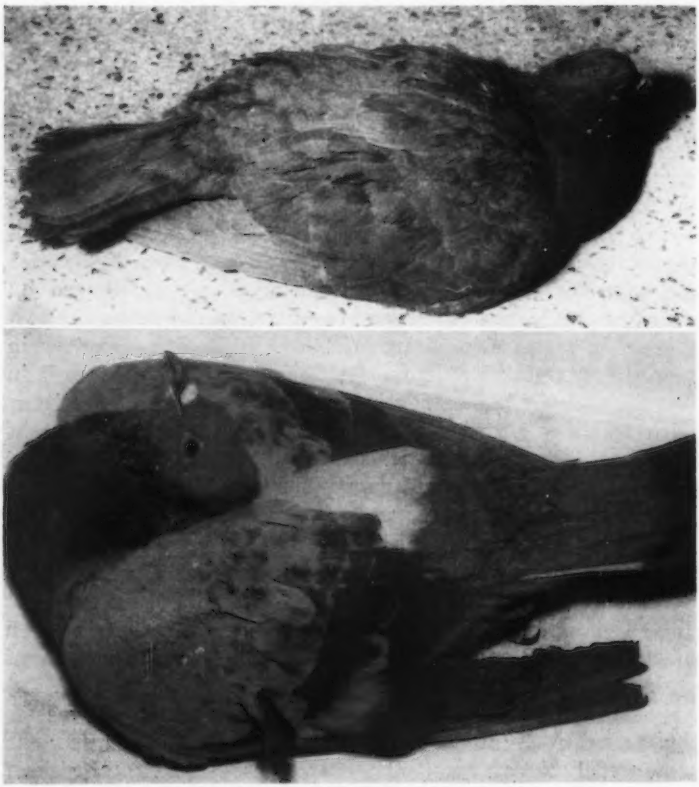

Fig. 1 (top). Acute toxicosis of dihydrostreptomycin sulfate in a pigeon. Prostration (inability to walk, lowered head, wings and tail) and somnolence (partial closure of eyelids) were characteristic findings. In most injected pigeons the head often was more extended and the eyelids more closed than is illustrated. The dose given this pigeon was 50 mg or approximately 125 mg/kg.

Fig. 2 (bottom). Acute toxicosis of procaine penicillin G in a pigeon. The characteristic opisthotonic spasm produced by 2,500,000 units/kg is shown.

in one of ten pigeons. Upon receiving the injection of dihydrostreptomycin two days later, this group also became somnolent. In some pigeons, slight ataxia was observed.

TABLE I

Acute toxicity of dihydrostreptomycin in pigeons. Each ml contained 0.4 gram of dihydrostreptomycin base, LD_{50} = 715 m./Kg.

Dose (ml)	Observations (Postinjection time intervals)
1000 mg/kg	
1.0	All 5 pigeons became somnolent and ataxic
0.95	in 8 to 15 minutes. They then became pros-
0.95	trate and comatose and died in 18 minutes
1.20	to 1 3/4 hours (avg. about 1 hour).
0.92	
800 mg/kg.	
0.80	Emesis in 1 pigeon in 5 minutes. All 5
0.80	pigeons became somnolent and ataxic in 5 to
0.76	15 minutes. They became prostrate, then
0.76	comatose and 4 died in 25 to 65 minutes (avg.
0.84	about 50 minutes). The remaining pigeon recovered in 1 3/4 hours.
630 mg/kg	
0.64	Emesis in 2 pigeons in 5 and 20 minutes. All
0.60	5 pigeons became somnolent and ataxic in 10
0.60	to 20 minutes. There was one death at 2 3/4
0.60	hours. The others were prostrate for several
0.60	hours and had recovered by the following morning.
500 mg/kg	
0.47	Emesis in 5 pigeons in 6 to 25 minutes. All
0.50	were somnolent and ataxic in 10 to 25 minutes.
0.47	They became prostrate or showed slight depression
0.47	for several hours and had recovered by morning.
0.40	
400 mg/kg	
0.35	Emesis in one pigeon in 30 minutes. All were
0.42	somnolent in 20 to 50 minutes. Two became
0.38	ataxic. Three recovered in 1 1/2 hours. The
0.40	other 2 showed slight depression for several
0.32	hours and had recovered by the following morning.

Group C: At the time of the second injection for Groups A and B, a third group (C) of pigeons received 0.2 ml of the antibiotic combination which contained 40,000 units of procaine penicillin G and 50 mg dihydrostreptomycin sulfate. All ten birds vomited or attempted to vomit in 15 to 25 minutes following the single combined injection. Emesis continued

TABLE II

Acute toxicity of procaine penicillin G in pigeons. Each ml contained 300,000 units of penicillin. LD_{50} = 1,750,000 units/kg.

Dose (ml)	Observations (Postinjection time interval)
2,500,000 units/kg	
3.2	Emesis in 4 to 15 minutes followed by opisthotonus in both pigeons. One pigeon died in 35 minutes and the second after several hours. The latter bird had watery diarrhea.
3.36	
1,000,000 units/kg	
1.32	Emesis in both pigeons at 14 and 45 minutes, respectively. Recovery at 50 minutes.
1.15	
400,000 units/kg	
0.47	Emesis in one pigeon in 32 minutes. No symptoms were observed in the other bird.
0.47	

intermittently in these birds for 10 to 15 minutes. Slight depression was observed in some birds but this finding was not so noticeable as a group as in pigeons in Groups A and B which received a single dose of dihydrostreptomycin.

Experiment III: The I. M. LD_{50} values of dihydrostreptomycin sulfate and procaine penicillin G in pigeons and chickens were determined as shown in tables 1, 2, 3 and 4. The LD_{50} of dihydrostreptomycin sulfate in pigeons was 715 mg/kg. The value was aproximately one-third that of the respective LD_{50} in chickens (2167 mg/kg). Acute toxicosis (figure 1, 3 and 4) of dihydrostreptomycin sulfate in both species was characterized by a brief initial ataxic stage followed by a depressive stage during which birds of these two species may be comatose for several hours unless otherwise terminated by death. In pigeons, emesis was a common occurrence just prior to and during the ataxic stage. Gasping at inspiration in four chickens during the depressive stage was considered noteworthy. It is of further interest that some of the chickens were slightly ataxic as long as 24 days after injection although some improvement did occur during that time.

TABLE III

Acute toxicity of dihydrostreptomycin in chickens. Each ml contained 0.4 gram of dihydrostreptomycin base. LD_{50} = 2167 mg/kg

Dose (ml)	Observations (Postinjection time interval)
2500 mg/kg	
3.53	One chicken died in 2 minutes. The other two showed
3.78	prostration and gasping and then became comatose and
3.9	died in 40 to 55 minutes.
2000 mg/kg	
*3.5	In 13 to 40 minutes, three chickens became ataxic,
*3.5	prostrate and then comatose for several hours.
^3.6	Except where noted below, they had recovered by the following morning.
1600 mg/kg	
2.08	In 15 to 35 minutes, this group became similar
*2.8	to the 2000 mg/kg group. One bird showed
2.4	inspiratory gasping. All recovered.
1250 mg/kg	
1.55	In 10 and 20 minutes, 2 chickens resembled those
*1.86	in the previous groups. No symptoms were observed
1.9	in the other chicken.
1000 mg/kg	
1.5	In 10 to 70 minutes, these chickens resembled those
1.5	in the above groups. One chicken showed inspiratory
1.5	gasping. One died 23 1/2 hours after injection (403).
800 mg/kg	
1.3	One chicken (402) died immediately (injection in blood
1.0	vessel?). The other two became prostrate in 25 minutes
1.3	and recovered in 4 hours.

* 24 days later, these four chickens were slightly ataxic (sl. incoordination of the legs, poor wing beat).

The LD_{50} of procaine penicillin G in pigeons was found to be 1,7500,000 units/kg. In two pigeons which received a 2,500,000 unit/kg dose, death was preceded by opisthotonic spasm, as shown in figure 2. As occurred following the dihydrostreptomycin sulfate injection, emesis was observed rather uniformly. Watery diarrhea was observed in one pigeon at the 2,500,000 units/kg. level.

TABLE IV

Acute toxicity of procaine penicillin G in chickens. Each ml contained 300,000 units of penicillin. LD_{50} = >2,500,000 units/kg.

Dose (ml)	Observations (Postinjection time interval)
2,500,000 units/kg	
5.08	Copious watery diarrhea in three chickens in 14 minutes. One chicken showed slight incoordination of the legs and somnolence for approximately one hour and recovered.
5.34	
4.91	
1,000,000 units/kg	
2.36	Copious watery diarrhea in three chickens in 15 to 25 minutes.
2.03	
1.60	
400,000 units/kg	
0.78	Watery diarrhea in three chickens in 30 to 40 minutes.
0.75	
0.83	

In chickens, the LD_{50} of procaine penicillin G exceeded 2,500,000 units/kg. The volume (>5ml) of a dose greater than this made further injection impractical. Watery diarrhea was the only consistent symptom of acute toxicosis of procaine penicillin g in chickens. One of the chickens showed transient ataxia at the 2,500,000 units/kg level.

No drug-induced gross lesions were observed on postmortem examination of all the birds under experiment.

DISCUSSION

As stated previously, an intramuscular injection of 40,000 units of procaine penicillin G and 50 mg of dihydrostreptomycin sulfate uniformly produced emesis in racing homer pigeons. No other symptoms were reported. In the present study, emesis was observed following the injection of the above dosage of either of these antibiotics. It is suggested that the apparently nonspecific propensity of the pigeon to vomit is an expression of a behavioral mechanism of this species as occurs with physiological regurgitation in feeding its young. It was also shown that the proneness toward emesis could be enhanced considerably by feeding just prior to injection.

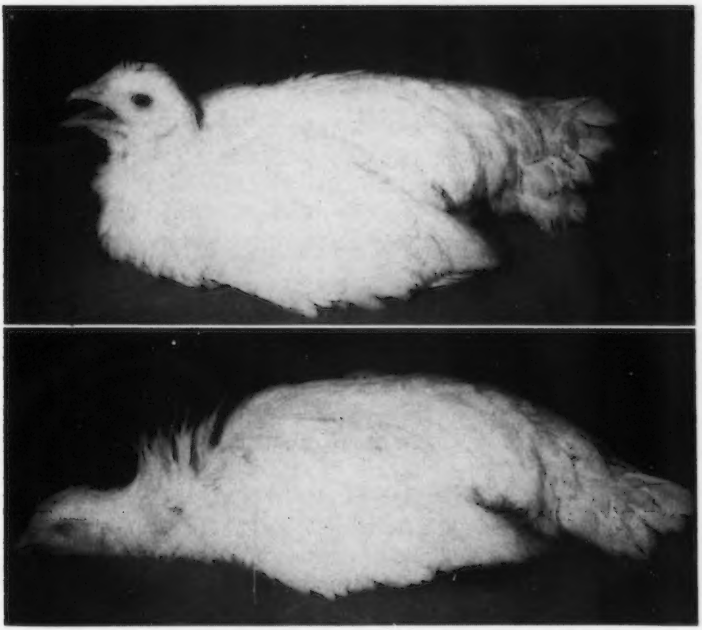

Fig. 3 (top). Acute toxicosis of dihyrostreptomycin sulfate in a chicken. Early depressive stage showing gasping and prostration. The dose was 2500 mg/kg.

Fig. 4 (bottom). Acute toxicosis of dihydrostreptomycin sulfate in a chicken. Late depressive or comatose stage in same chicken as shown in figure 3.

Toxic manifestations such as ataxia, labored breathing and coma which were observed in one or both of the species used in the present study were similar to those mentioned by Jones[2] as characteristic of acute streptomycin poisoning of mammalian species. The results indicated that pigeons are more susceptible to the acute toxic effects of both of the antibiotics which were tested than are chickens. This finding was determined by the more severe symptoms in the pigeon when the two species were given comparable dosages and by the lower LD_{50} values obtained for the pigeon. Although the

minimal toxic doses of these antibiotics were not established for pigeons, the following dosages are regarded as nontoxic:

Dihydrostreptomycin-80 mg/kg or 25 to 30 mgs for an adult pigeon.

Procaine penicillin G-80,000 units/kg or 30,000 units for an adult pigeon. It would be desirable to inject pigeons several hours before or after feeding so as to minimize the possibility that emesis may occur. The effectiveness of the above dosages in the control of chronic respiratory disease in pigeons has not been established.

SUMMARY

Acute toxicosis of dihydrostreptomycin sulfate in pigeons was characterized by ataxic and depressive stages. The intramuscular LD_{50} was 715 mg/kg.

An intramuscular dose of 2,500,000 units/kg of procaine penicillin G, caused opisthotonic spasm in two pigeons. The LD_{50} was 1,750,000 units/kg.

The proneness of pigeons to emesis was considered to have relatively non-specific toxicological significance.

In chickens, the signs of acute toxicosis of dihydrostreptomycin sulfate were similar to those in the pigeon. The IM LD_{50} was 2167 mg/kg.

Intramuscular injections of 40,000 units/kg and higher of Procaine Penicillin G produced watery diarrhea in chickens. The LD_{50} was $>2,500,000$ units/kg.

REFERENCES

1. Crawley, J. F., and J. E. Fahey. Poultry Science, *34*, 707, 1955.
2. Jones, L. M. Veterinary Pharmacology and Therapeutics. 1st ed. Ames, Ia.: Iowa State College Press, 1954.

A TISSUE CULTURE-MODIFIED NEWCASTLE DISEASE VIRUS
I. MODIFICATION, PROPAGATION AND CHARACTERISTICS OF THE TISSUE CULTURE NEWCASTLE DISEASE VIRUS

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IT HAS been realized for some time that the chronic respiratory disease conditions in chickens are frequently due to a mixture of infectious agents.[12,14,15] Pathogens of poultry are not always introduced as field infections but may be introduced through vaccines which are prepared from virulent strains of the organism which, on application, may introduce and perpetuate the disease in fully or partially susceptible birds. In other instances the vaccine strains of the virus may be superimposed on existing disease conditions that may result in a serious respiratory disease complex. The respiratory disease complex cannot be solved with the control and elimination of one infectious agent. Investigations towards improving products for immunization so as to prevent introduction and spread of a live virulent agent as a potential synergist in the respiratory disease complex, is believed to be the logical approach.

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Since present inactivated vaccines have been demonstrated to induce an inadaquate immunity particularly in laying hens, studies were concentrated on producing a live modified virus propagated in tissues other than embryonating chicken eggs. Cultivation of the virus in vitro is considered important to prevent the possibility of introducing egg-borne infections to poultry flocks through vaccines which are prepared from fertile hen eggs.[9,10,11,13,16]

Herein is described a modification of a Newcastle disease virus (NVD) which, upon injection into chickens of any age, produced an immunological response without inducing clinical manifestations of the disease.

MATERIALS AND METHODS

Virus: Propagation and serial passage of the California 11914 strain of Newcastle disease virus in test tubes which contained minced chicken embryos suspended in Simm-Sanders media (SS)† that resulted in change of virulence but retained immunogenicity has been described.[2] Suspensions prepared with the 47th through the 55th serial passage of the virus, administered to chickens by the air-borne route in an enclosed chamber, gave encouraging results. However, on occasion paralytic symptoms were produced in as high as 6.6 percent of exposed birds. It also was lethal to very young chicks (48 hours old) when given parenterally.[1] To eliminate the adverse effects from the clone, the virus was further studied by manipulation with laboratory procedures.

Titration: The virus during attenuation showed a progressive loss of virulence for chickens. However, it was consistently lethal for embryonating eggs. All titrations of the tissue culture propagated NDV were conducted in 8 to 11-day-old chicken embryonated eggs, inoculated with 0.1 ml into the allantoic sac. The time of death following inoculation was about 64 hours; however, embryos inoculated

† The media was composed of 80% Simm-Sanders salt solution, 18% bovine serum ultrafiltrate, to which was added 1% of a saturated solution of p-amino benzoic acid and 1% of a 5 mg percent solution of cysteine hydrochloride. The cultures were incubated for 72 hours at 37 C and 24 hours thereafter at room temperature.

with the higher concentrations of virus were killed by the 48th hour. The hemagglutination (HA) activity of the allantoic and amniotic fluids (aafl) and lesions upon death were not unlike those observed following inoculation with the original NDV suspension.

Serology: The hemagglutination-inhibition (HI) and serum neutralization (SN) tests were conducted according to the methods described by Beach.[7] The HI test was modified by decreasing the red blood cell suspension from 1.0 to 0.75 percent. Experience in this laboratory had shown that birds with an HI titer of 1:80 resisted an intramuscular challenge of at least 200,000 embryo minimum lethal doses (mld) of virulent virus. An HI titer of 1:40 corresponded to a similar immunity in 95 percent of the birds.

The geometric mean titer (GMT) was used in place of the arithmetic mean to demonstrate more adequately a mean HI response in a group. The GMT was computed by first dividing the reciprocal of the HI titers by 10 and each titer expressed as the logarithm to the base 2. The sum of these values was calculated and the mean obtained by dividing by the total number of sera in the group. The antilogarithm (base 2) of this mean was multiplied by 10 to give the GMT.

Example:

No. Samples (f)	reciprocal of HI titer ÷ by 10	log base 2	X f
5	neg	—	—
6	1.0	0	0
3	4.0	2	6
6	8.0	3	18
20			24

$$24 \div 20 = 1.2 \text{ antilog} = (2)^{1.2} = 2.29$$
$$\text{GMT} = 2.29 \times 10 = 22.9$$

The G.M.T. is an expression which is used most efficiently for positive titers.[8] In this report the sum of the logarithms of the titers were divided by the total number of sera including the HI negative samples in the group. When no HI

antibodies were demonstrated in sera of all individuals in a group, the GMT was expressed in the tables as "negative". In no case were HI antibodies detected in controls or in susceptible birds placed in contact with vaccinated chickens.

Challenge Virus: The GB-Texas 1948 or California "C" strains of NDV were used for all challenges. Each of the strains were propagated in chicken embryonating eggs, and the aafl were pooled, distributed in 2 ml ampoules, and stored at —6 C. The pool was titrated in chicken embryonating eggs at 4 week intervals to ensure constancy of titer. The challenge dose given by intramuscular (IM) and air borne (AB) routes were in all cases 2×10^5 embryo mld unless otherwise indicated.

PROCEDURE AND RESULTS

Serial passage through media with and without bovine ultra filtrate. To eliminate the paralytic and lethal factor from the suspension, a series of experiments were conducted with the 47 serial passages harvested in September of 1951 through SS media containing bovine ultrafiltrate. The suspension was inoculated into several test tubes containing SS media without ultrafiltrate for two passages to increase the chicken embryo titer†. Table 1 illustrates the series of alternate passages of the virus through media with and without bovine serum ultrafiltrate. The series was carried for 16 passages beyond the original 47 passages constituting 63 serial passages from the original inoculum (OS63). It was observed the chicken embryo titers of the cultures varied from 10^3 to 10^7; however, more consistent titers were obtained when the passages were conducted more frequently and with regularity.

Back passage of the alternate series through chicken embryonating eggs. To determine whether the above treatment eliminated the paralytic factor when administered to growing chickens, the 10th passage of the alternate series (OS 57) was serially back passed through chicken embryonating eggs. The aafl of the 1st, 10th, 20th, 30th, 40th and

† It was previously established that the virus could be propagated in cultures without serum ultrafiltrate.

TABLE I

Titers of Newcastle disease virus passed alternately through Simms-Sanders medium with and without bovine serum ultrafiltrate

Serial passage from original virus (OS)	Passage number	Titer of culture propagated		Days between passages
		With BUF †	Without BUF †	
47				
48	1	. . . .	n d	25
49	2	. . . .	n d	4
50	3	10^5	. . . .	24
51	4	. . . .	10^6	22
52	5	10^6	. . . .	22
53	6	. . . .	n d	114
54	7	10^7	. . . .	15
55	8	. . . .	10^7	13
56	9	10^3	. . . .	69
57	10	. . . .	n d	174
58	11	10^4	. . . .	10
59	12	. . . .	10^5	115
60	13	10^5	. . . .	4
61	14	. . . .	10^6 or>	4
62	15	10^7	. . . .	4
63	16	. . . .	10^5	83

† BUF — bovine serum ultrafiltrate
n d — not done

50th passages were harvested, sealed in ampoules and stored at —6C until the passages were completed. The affl were thawed and adjusted, following titration in embryonating eggs, to contain 10^6 mld in 0.1 ml of suspension.

Each of six birds, 7 weeks of age, were inoculated intramuscularly with 0.25 ml of each of the six inoculums. The results of this trial (table 2) showed that one or two birds of each of the groups inoculated with the 10th, 20th, 30th, 40th, and 50th serial back passages through eggs showed some signs of nervous system involvement. One bird in the group inoculated with the 40th passage died as a result of inoculation. The others showed symptoms of paralysis involving one of the legs. On the 12th day after inoculation, HI titers of the asymptomatic birds ranged from 1:40 to 1:320. There was no correlation in the height of HI titer with the successive back pass material. All remaining birds were refractory to an intramuscular challenge with a virulent virus 12 days following inoculation.

Birds with paralytic symptoms, that developed between

TABLE II

Results of inoculating 7-week-old chickens with allantoic and amniotic fluids of embryos serially inoculated with the 57th passage NDV

Passage number in embryos	Clinical signs[1]	Immune response 12th day following inoculation	
		HI titer (range)	Challenge results of survivors[2]
1	0/6	40-80	0/6
10	2/6[P]	160-320 or>	0/4
20	2/6[P]	80-320 or>	0/4
30	1/6[P]	40-320 or>	0/5
40	3/6 [2P 1D]	40-320 or>	0/3
50	1/6[P]	80-320 or>	0/5
Controls uninoc.	0/4	Neg	4/4

[1] — No. with signs / No. inoculated [P] — Paralyzed [D] — Dead
[2] — 100,000 eld of strain C Newcastle disease virus given intramuscularly, no. suscept./no. inoculated.

the 7th and 11th days following inoculation, were immediately killed and spleens and brains of each bird pooled and serially passed through embryonating eggs at least three times. In no instance was the virus isolated from the birds.

Rapid passage of tissue culture (TC) NDV. Inasmuch as the tissue culture suspension still appeared to contain the undesirable factor, Sabin's method of rapid passage was attempted. Using the 16th alternate passage (OS 63) with an embryo mld titer of 10^5 was inoculated into SS media without serum ultrafiltrate. The volume of inoculum for this and each succeeding passage was 20 percent of the total volume of the culture. No serum ultrafiltrate was used in the media employed in this series. The passages were continued at 24-hour intervals for eight serial passages. The next two passages (72nd and 73rd) were incubated for 96 hours to obtain a sufficient quantity of virus for testing and then the series was continued with a 24-hour incubation period (figure 1).

To determine the effect of this procedure upon the clone, the 9th passage of the rapid series (OS 72) was inoculated

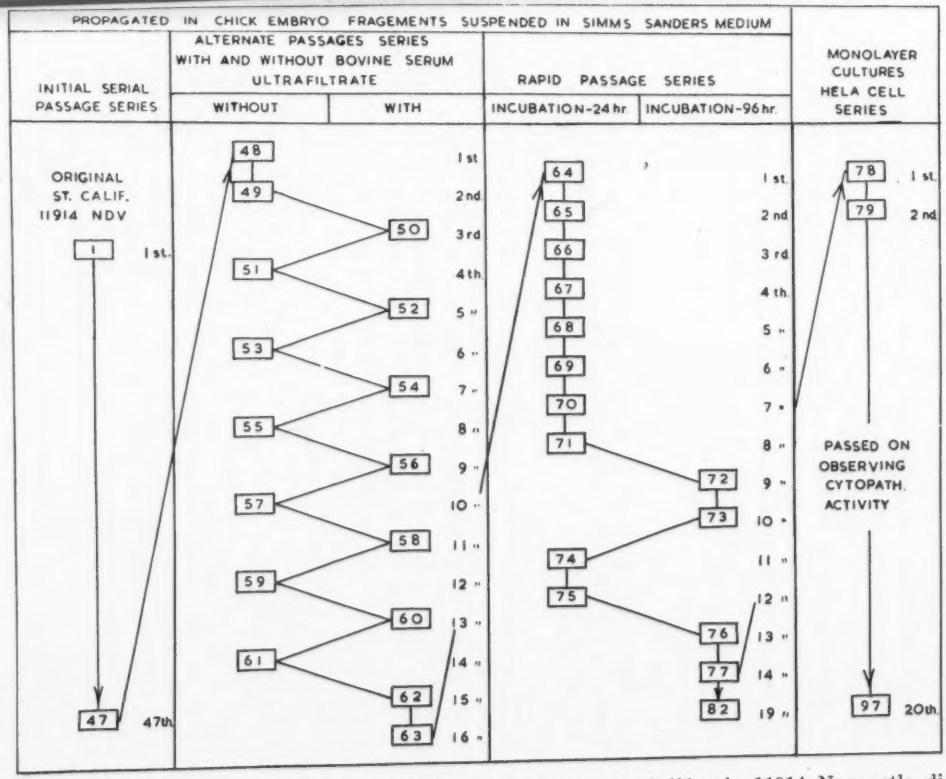

Fig. 1. Schematic presentation of serial passage of strain California 11914 Newcastle disease virus through tissue culture.

into 130 birds of 12 days of age. The embryo mld titer of the virus was 10^5 per 0.1 ml and 0.25 ml of the culture was given intramuscularly. No symptoms of Newcastle disease involving the respiratory or nervous systems were observed. HI titers on the 13th day after inoculation of 20 chicks ranged from 0 to 1:80. Fourteen birds had no HI titer, one bird had a titer of 1:10, three birds had a titer of 1:20, one bird had a titer of 1:40 and one bird had a titer of 1:80. All birds were refractory to an intra-muscular dose of 100,000 embryo mld of strain GB NDV. The four uninoculated control birds were susceptible and died after exposure to the virulent virus by the 5th day.

Since no symptoms were observed in the 12-day-old birds, it was believed advisable to determine further whether the paralytic factor was eliminated by this technique. Larger quantities of the virus using the 96 hour incubation period were employed to insure higher titers of the virus. Using the 12th passage (OS 75) the series was regularly passed at 96 hour intervals and continued through 19 passages (figure 1).

Back passages of the rapid series in chicken embryonating eggs. Using the 19th passage of the rapid series (OS 82), 50 passages were made through chicken embryonating eggs. The aafl of the 10th, 20th, 30th, 40th, and 50th serial passage levels were collected and stored at 4 C until this series was completed. Each of the fluids was titrated and found to have an infectivity titer of 10^6 or 10^7 embryo mld. Each of the fluids was adjusted by dilution to contain 10,000 embryo mlds of virus. The latter suspensions were inoculated in doses of 0.25 ml intramuscularly into each of six 7-week-old susceptible chicks. None of the chicks showed any evidence of Newcastle disease during a 15-day observation period, and all birds were refractory to intramuscular challenge of 200,000 embryo mld of challenge virus. These results were sufficiently encouraging to justify more extensive trials. Virus propagated in chicken embryo tissues after the 12th serial passage in this series was designated as line TC and the characteristics and immunogenic properties of the strain are described elsewhere.[3,4]

Effect of the virus inoculated intravenously and intracerebrally. Table 3 shows the results of two groups of 10 chickens each, 47 days of age which were inoculated intravenously with 0.5 ml of the OS 82nd passage of the rapid series. Group Ia was inoculated with a suspension which contained 10^5 embryo mld of the virus and Group I was inoculated with a suspension containing 10^4 embryo mld of the same suspension. Group III which was composed of hatchmates of Groups Ia and Ib were controls for the entire experiment.

TABLE III

Results of inoculating chickens intravenously and intracerebrally with the 82nd passage Newcastle disease virus propagated in tissue cultures using chick embryo tissues

							Immune response		
			Inoculum				Titers 4th wk		Challenge 5th wk[2]
Group	No. chicks	Age (days)	Route	Titer	Dose ml	Signs and mortality	HI GMT	SN[2]	
I a[1]	10	49	I V	10^5	0.5	0/10	49	10^3 10^3	0/10
I b[1]	10	49	I V	10^4	0.5	0/10	21.	10^1 10^3	0/10
II a	25	1	I C	10^5	.05	12/25	10.	10^1 $<10^1$	0/13
II b	25	1	I C	10^4	.05	11/25	10.3	$<10^1$ $<10^1$	0/14
III[1]	7	10				0/7	Neg	$<10^1$ $<10^1$	7/7

[1]—8 birds picked at random before inoculation were negative to HI and SN tests and all birds were susceptible to challenge of 200,000 mid of ND virus.
[2]—Equal volumes of 4 sera in each of two pools.
[3]—200,000 mid of strain GB virus intramuscularly.

No respiratory or nervous symptoms of Newcastle disease or any other untoward reactions were observed in the inoculated birds during the 5 week observation period. High HI and SN titers were found 4 weeks after inoculation. Although HI and SN titers were observed in birds receiving

the greater quantity of virus, the titers were no greater than could be expected following an intramuscular injection. All birds were refractory to an IM challenge of strain GB NDV 5 weeks after inoculation.

The one-day-old chicks were divided into two groups and inoculated intracerebrally with the same suspensions used for Groups Ia and Ib. Approximately one-half of the chicks inoculated with either dilution of the virus were affected. Symptoms of depression were noted by the 4th or 5th day which were followed in a few hours by leg weakness. Progressive paralysis and clonic spasms developed, followed by death by the 7th or 8th day. The asymptomatic chicks were held until the 5th week and bled for the HI and SN test. Only two chicks (one in Group IIa and one in IIb) had presented HI titers of 1:10 and 1:20 while the remaining chicks showed no detectable HI antibodies. All of the asymptomatic birds were immune to intramuscular challenge 5 weeks following inoculation.

Propagation of the virus in Hela cell cultures. Using the OS 77 passage, the virus was propagated for 20 generations in monolayer cultures of Hela cells and designated as line TCH^5. To determine the immunogenicity of the virus grown in Hela cell cultures, the 20th passage (OS 97) was compared with the OS 76th passage of the virus propagated in fragments of chicken embryo tissue. Each of the virus suspensions were titrated in embryos and diluted with buffered saline (ph 7.4) to contain 10^4 embryo mld infectious doses in 0.1 ml. Table 4 shows the results of each of two groups of 20 chickens, 47 days of age, inoculated intramuscularly with 0.2 ml of each of the two lines of virus suspensions. The results indicated that a comparable immunological response was obtained with each line of virus and all birds were refractory to intramscular challenge 12 weeks later.

DISCUSSION

It is beyond the scope of this paper to determine the nature or mechanism of mutation of the virus; however, attenuation of the California 11914 strain of NDV was accomplished by serial passage through tissue culture. The

TABLE IV

Immune response of 47 day old chickens to the attenuated Newcastle disease virus propagated in chicken embryo and Hela cell tissue cultures

Group	Dose intra-musc. (ml)	Line and passage level of virus[1]	No. of Birds	Immune Response[2]						
				Prior inoc.		3 wks after inoc.		12th week after inoc.		
				HI (GMT)	SN	HI (GMT)	SN	HI (GMT)	SN	Challenge
1	0.2	76th T C	20	Neg.	$<10^1$	32	10^1	Neg.	$<10^1$	0/20
2	0.2	95th TCH	20	Neg.	$<10^1$	25	10^1	11.5	10^1	0/20
3	—	None	20	Neg.	$<10^1$	Neg.	$<10^1$	Neg.	$<10^1$	20/20

[1]—Line T C = virus propagated in chicken embryo tissues: TCH = virus propagated in monolayer culture of Hela cells

[2]—SN titer = arithmetic mean of 5 individual sera tested. All birds challenged intramuscularly with 200,000 embryo lethal doses of strain GB NDV, No. Susceptible/No. challenged.

latter was composed of fragments of chick embryos suspended in a modified Simm-Sanders medium containing bovine serum ultrafiltrate. Although 55 serial passages resulted in a virus which on occasion produced paralysis or death, the use of the Sabin method of rapid passage in large quantity resulted in an attenuated strain of virus. The latter upon intramuscular injection in young chicks did not induce symptoms of the disease but provided an immunity which resisted challenge of large doses of virulent ND virus.

Other changes in tropism of the modified virus were the notable decrease in HI titer response of parenterally inoculated chickens and the apparent inability or difficulty of isolation of the virus from birds inoculated with TC or aafl of embryos infected with the TC line of virus.

The original strain of virus used for attenuation was highly virulent for both embryos and chickens. This strain has been used in this and other laboratories for a number of years as the strain of choice for challenge purposes. Following intramuscular injection, symptoms of clonic spasms, paralysis, followed by death which approached 100 percent was consistently obtained. The attenuated strain did not induce signs in the respiratory or nervous systems following large doses given intramuscularly or intravenously. Intracerebral inoculation of one day old chicks resulted in mortalities of 44 to 48 percent.

Although the attenuated virus did not produce symptoms in chickens, embryonating eggs of hens were readily susceptible. This provided a convenient means of titrating the virus concentrations.

The virus reported herein showed that it can be used as a vaccine since it does not cause clinical signs of ND in chickens nor does it regain its virulence for chickens following 50 serial passages in chicken embryonating eggs. Laboratory and field trials with the virus over the past two years have confirmed these findings.[3,4]

An advantage of the vaccine strain is that while it can be propagated in tissues of chicken embryos it is also readily grown in cultures of Hela cells. Growth in bovine and swine

kidney cell tissue cultures have also been demonstrated.[5,12] The propagation of the virus in tissues other than the chicken embryonating egg reduces the possibility of introducing pathogens for chickens which may be present in embryonating hen eggs used for vaccine production.

SUMMARY

The California 11914 strain of NDV was attenuated by serial passage through Simm-Sanders medium containing fragments of chicken embryo tissue and bovine serum ultrafiltrate.

1. The attenuated virus did not produce signs of Newcastle disease in susceptible chickens when given intramuscularly or intravenously.

2. The virus was highly pathogenic for chicken embryos but did not regain its virulence for chickens after 50 serial passages through the susceptible host tissue.

3. The virus propagated in chick embryo tissues or in monolayer cultures of Hela cells induced a high immunogenic response.

REFERENCES

1. Bankowski, R. A., Vet. Med., *95*, 1950.
2. Bankowski, R. A. and W. Boynton, Vet. Med. *43*, 305, 1948.
3. Bankowski, R. A., R. Corstvet, J. Fabricant. Avian Dis. in press.
4. Bankowski, R. A., R. Corstvet, J. Fabricant. Avian Dis. in press.
5. Bankowski, R. A. and J. Hyde. Am. Jour. Vet. Res. *18*, 743, 1957.
6. Beach, J. R. Proc. U.S. Livestock Sanitary Assoc. *48*, 177, 1945.
7. Beach, J. R. Jour. Am. Vet. Med. Assoc. *112*, 85, 1948.
8. Fryer, N. C. John Wiley & Sons Inc. N.Y., 1957.
9. Gottral, H. Ann N. Y. Acad. Sci. *55*, 221-234, 1952.
10. Hofstad, M. S. Vet. Med. Res. Inst. Iowa State College *5*, 1957.
11. Thompson, C. H. Am. Jour. Vet. Res., *15*, 293, 1957.
12. Unpublished data.
13. U.S. Dep. Ag. Mimeo 52, 71-72, 1952.
14. Sullivan, J. F. Avian Dis. *1*, 94-100, 1957.
15. Van Roekel H., J. E. Gray, N. L. Shipkowitz, M. K. Clarke, R. H. Luchini. Univ. of Mass. Bull. 486, 1957.
16. Zagar, S. L., B. S. Pomeroy. Jour. Am. Vet. Med. Assoc. *116*, 304, 1950.

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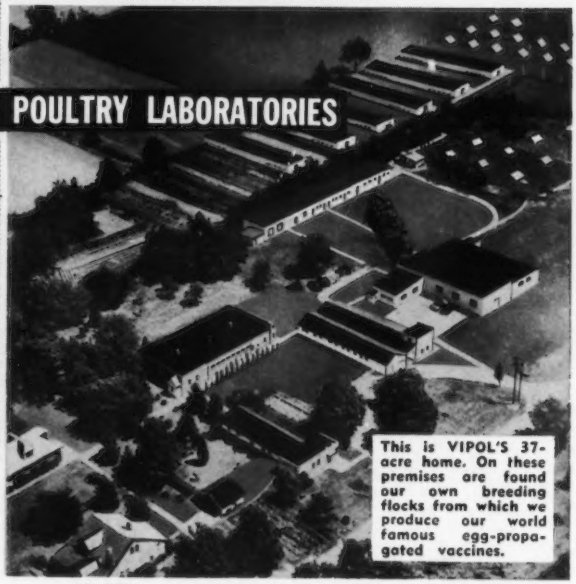
MADISON 1, WISCONSIN

VINELAND POULTRY LABORATORIES



This is VIPOL'S 37-acre home. On these premises are found our own breeding flocks from which we produce our world famous egg-propagated vaccines.

First and Foremost in Protecting Poultry Health!

SINCE 1914, Vineland Poultry Laboratories has been uncompromising in its policy of producing only the highest quality vaccines and other biologics for the American poultry farmer. Our products have had as one of their principal goals the earning of the approbation and respect of veterinarians, poultry pathologists and others dedicated to the best interests of agricultural production.

Our mission has been and is being accomplished. But, even as we continue with our laboratory research and field testing, we are ever mindful that our work must meet the unshakable requirements of poultry scientists. We pledge to continue striving toward this end.

Ask For It!

Complete literature for poultry pathologists, laboratory technicians and others engaged in poultry research is available in any quantity on request.

VINELAND POULTRY LABORATORIES

VINELAND, NEW JERSEY

RESEARCH

made . . .

DR. SALSBURY'S

WORMAL

GRANULES AND TABLETS

A COMPLETE TREATMENT FOR POULTRY WORMS

Wormal removes all three of the economically important types of worms from chickens and turkeys: (1) Tapeworms – six commonest species; (2) Large roundworms, and (3) Cecal worms. Wormal is most effective for poultry worm removal because –

Only WORMAL contains all three of these drugs

DIBUTYLTIN DILAURATE
PIPERAZINE
PHENOTHIAZINE

Intensive research resulted in the use of Dibutyltin dilaurate, combined with Piperazine and Phenothiazine to make Wormal. This combination has now been proved safe and effective on millions of birds.

Wormal *granules* mix easily in the feed for flock treatment; Wormal *tablets* make individual treatment practical. Economical as well . . . one egg saved per bird pays for a treatment.

These are the simple facts but we will be pleased to give you more detailed, authoritative data if you'll write . . .

DR. SALSBURY'S LABORATORIES
Charles City, Iowa

Dr. SALSBURY'S

nation-wide service to the poultry industry

Continuing Leadership in Coccidiosis Control!

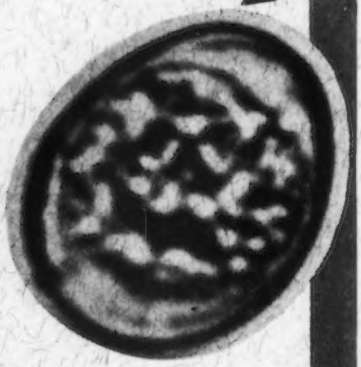
1948...S.Q.®
1954...NICARB®
and in 1958...

SAFE · POTENT · EFFECTIVE

GlyCamide*

(glycarbylamide)

The newest member of the Merck Coccidiostat family



THE VALUE of a coccidiostat is measured by these criteria: feed conversion ratios; shank and feather pigmentation; absence of clinical symptoms or coccidiosis mortality (under a wide range of exposure levels); and a high degree of resistance to reinfection. GLYCAMIDE has *demonstrated all* of these attributes as an anticoccidial agent for broilers and for replacement chicks raised for laying or breeding flocks!

Potent GLYCAMIDE is the new anticoccidial agent from Merck, for the prevention of cecal and intestinal coccidiosis in growing chickens. When incorporated in the feed at a 0.002% level for replacement flocks and a 0.003% level for broilers, this compound is highly

Effective against the common pathogenic species of coccidia: *Eimeria tenella, E. necatrix* and *E. acervulina.* In spite of its high potency in suppressing oocyst development (2nd generation schizonts and merozoites are principally affected), GLYCAMIDE has been proved remarkably

Safe even when fed at 100 X recommended preventive levels to growing chickens. GLYCAMIDE also has been fed to laying hens at the 0.003% level with no effect on fertility, hatchability, egg production, or internal and external egg quality.

MERCK

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CHEMICAL DIVISION
RAHWAY, NEW JERSEY

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